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A STUDY OF DOPA DECARBOXYLASE IN THE FLESHFLY, *SARCOPHAGA BULLATA*

by

© THOMAS T. CHEN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF GENETICS

EDMONTON, ALBERTA

FALL, 1973

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "A Study of Dopa Decarboxylase in the Fleshfly, *Sarcophaga bullata*," submitted by Thomas T. Chen in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

In this thesis, the biochemical and biological properties of dopa decarboxylase in *Sarcophaga bullata* are described. Dopa decarboxylase requires pyridoxal-5'-phosphate as an obligatory co-factor, and the rate of decarboxylation is also elevated slightly by Fe^{+++}. The enzyme is labile at 4°C, but sulfhydryl compounds and pyridoxal phosphate can stabilize the enzyme to a certain extent. The optimum pH for dopa decarboxylase activity in 0.05 M Tris-HCl buffer is 7.5, and the optimum assay temperature in this buffer is 35 - 40°C. The apparent molecular weight of the enzyme, determined by Sephadex G-200 column chromatography, is $5.1 \pm 0.3 \times 10^4$ A.M.U.

Three distinctive peaks of dopa decarboxylase activity are observed during the postembryonic development of *Sarcophaga bullata*. These peaks occur at pupariation (peak I), 5-1/2 days after pupariation (peak II) and at eclosion (peak III). The activity in peak I is confined to the epidermal cells. The DEAE cellulose chromatographic properties of the enzyme extracted from the epidermal cells of animals 12 hr after pupariation are similar to those of the enzyme found in the heads of 10 day old adults.

The activity of dopa decarboxylase can be induced in young third instar larvae (5 day old) by the injection of 20 µg ecdysterone. About 60% of the induced enzyme has the same DEAE cellulose chromatographic properties as that from the cuticular epidermal cells of brown puparia.

An *in vitro* system for studying the hormonal induction of the

enzyme was developed. Wing discs cultured *in vitro* for 3 days retain appreciable rates of RNA and protein synthesis. Such discs can undergo evagination when ecdysterone (β-ecdysone), ranging from 2×10^{-7} - 1×10^{-5} M, is added. Concentrations of α-ecdysone ranging from 6×10^{-6} - 2×10^{-5} M failed to induce evagination. The instantaneous rates of RNA and protein synthesis in imaginal wing discs are stimulated by the addition of ecdysterone. This ecdysterone-induced RNA and protein synthesis is inhibited by actinomycin D and cycloheximide concentrations which effectively block the evagination of the discs.

The activity of dopa decarboxylase can be induced in cultured imaginal wing discs. A 4-fold increase in enzyme activity over the control is observed after 96 hr in the presence of ecdysterone. The induction process is sensitive to actinomycin D and cycloheximide. The establishment of this system will allow further biochemical studies on the molecular mechanisms of the hormonal regulation of gene expression.

ACKNOWLEDGEMENTS

The author would like to express his great appreciation for the guidance and constructive criticism he received from Dr. R. B. Hodgetts, without whose help this study would not have been possible. Appreciation is also extended to Drs. A. Ahmed, M. Watanabe, B. Heming and M. Russell for their valuable comments during the period of this work.

A special word of thanks is due to the Department of Genetics, University of Alberta, for supplying materials and facilities necessary for the study undertaken here.

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Chapter I

INTRODUCTION

As a multicellular organism develops, there is a progressive restriction in the spectrum of differentiated cell types to which any cell can give rise. The idea (see Davidson, 1969, for discussion) that this restriction in the developmental potentialities of a cell was accompanied by irreversible inactivation of the genome, was effectively ruled out by the nuclear transplantation studies of Gurden (1963). In this work, nuclei from fully differentiated intestinal cells of *Xenopus* were shown to possess the complete spectrum of genetic potential that resides in the zygote. A second suggestion that differentiation results from differential replication of the genome during development (see Ebert and Kaighn, 1966, for review), has now been eliminated by the DNA-DNA hybridization competition experiments performed by McCarthy and Hoyer (1964). These authors found that there were no differences in the DNA sequences between embryonic and differentiated somatic cells. The mechanism which has received most experimental support is the selective release of gene activity resulting from the differential transcription of the genome (Britten and Davidson, 1969). As defined by Allen (1965) then, "Differentiation is the outward and visible sign of selective gene activity, the reflection of a change in the cell's biochemical repertoire as a consequence of the release of information encoded in one-dimensional sequences." Thus, the diversity of proteins which arises during development as a result of differential gene activity is thought to be responsible for cellular differentiation.

Among those factors which affect the selective release of gene activity, hormones are believed to be one of the most important. Several studies on the induction of specific protein synthesis by hormones have been reported, and among them, two systems have been studied extensively. The first of these is the synthesis of ovalbumin induced by estrogen, a female sex hormone, in immature chick oviducts (O'Malley et al., 1969, for review). Although extensive *in vivo* biochemical studies have been performed, the lack of a proper *in vitro* system is a drawback. The second example of hormone regulation of enzyme synthesis is the induction of tyrosine amino transferase by glucocorticoid hormones in hepatoma tissue culture cells (Tomkins et al., 1969; Tomkins and Gelehrter, 1972). Since these studies were performed on a tumor cell line, the extension of their results to normal cells is uncertain. Most importantly, though, neither of these two systems lends itself to genetic analysis. For these reasons, the development of other systems for studying the mechanism of hormone action is desirable.

The remarkable metamorphosis that insects undergo has long attracted the attention of developmental biologists. The morphological changes associated with insect metamorphosis are regulated by various hormones (Wyatt, 1972). In particular, sclerotization, the hardening and darkening of the insect cuticle during pupariation, is one of the best examples. This process is clearly under the control of the molting hormone, ecdysone (Gilbert and Schneiderman, 1961). Since the biochemistry of sclerotization has in large part been worked out by Karlson and his co-workers (Karlson and Sekeris, 1964), it is a favourable model system for studying the action of hormones. Furthermore, the availability of

sophisticated genetic techniques in one of the *Diptera*, namely *Drosophila melanogaster*, allows for the introduction of a genetic analysis. At the time this work was initiated though, several technical problems prevented studies on *Drosophila* and the fleshfly, *Sarcophaga bullata*, was chosen instead. This thesis describes the properties of dopa decarboxylase, one of the enzymes involved in sclerotization, and its regulation by the molting hormone ecdysone.

(A) Sclerotization and Tyrosine Metabolism in Higher Diptera

Much of the biochemistry of sclerotization has been worked out by Karlson and his co-workers for blowfly *Calliphora erythrocephala* (Karlson and Sekeris, 1964). In the late third instar larvae of *Calliphora*, tyrosine, instead of being metabolized through a degradative pathway, is converted to N-acetyl-dopamine by oxidation, decarboxylation and acetylation (Fig. 1.1) (Karlson and Sekeris, 1964). During pupariation, N-acetyl-dopamine is oxidized to an o-quinone by phenol oxidase, and the o-quinone then supposedly interacts with cuticular protein to harden the larval cuticle (Karlson and Sekeris, 1964; Brunet, 1965). Other studies such as those of Summers (1968) on *Uca pugilator*, Bodnaryk (1970, 1971 a,b) on *Sarcophaga bullata*, and Hodgetts and Konopka (1973) on *Drosophila melanogaster* suggested that with some minor modifications, this biochemical pathway is similar for a wide variety of organisms.

Ligation of *Calliphora* larvae prior to puparium formation, so as to isolate the ring gland in the anterior part of the animal, prevents sclerotization of the posterior part of the cuticle (Gilbert and Schneiderman, 1961; Karlson and Sekeris, 1964). Sclerotization of the

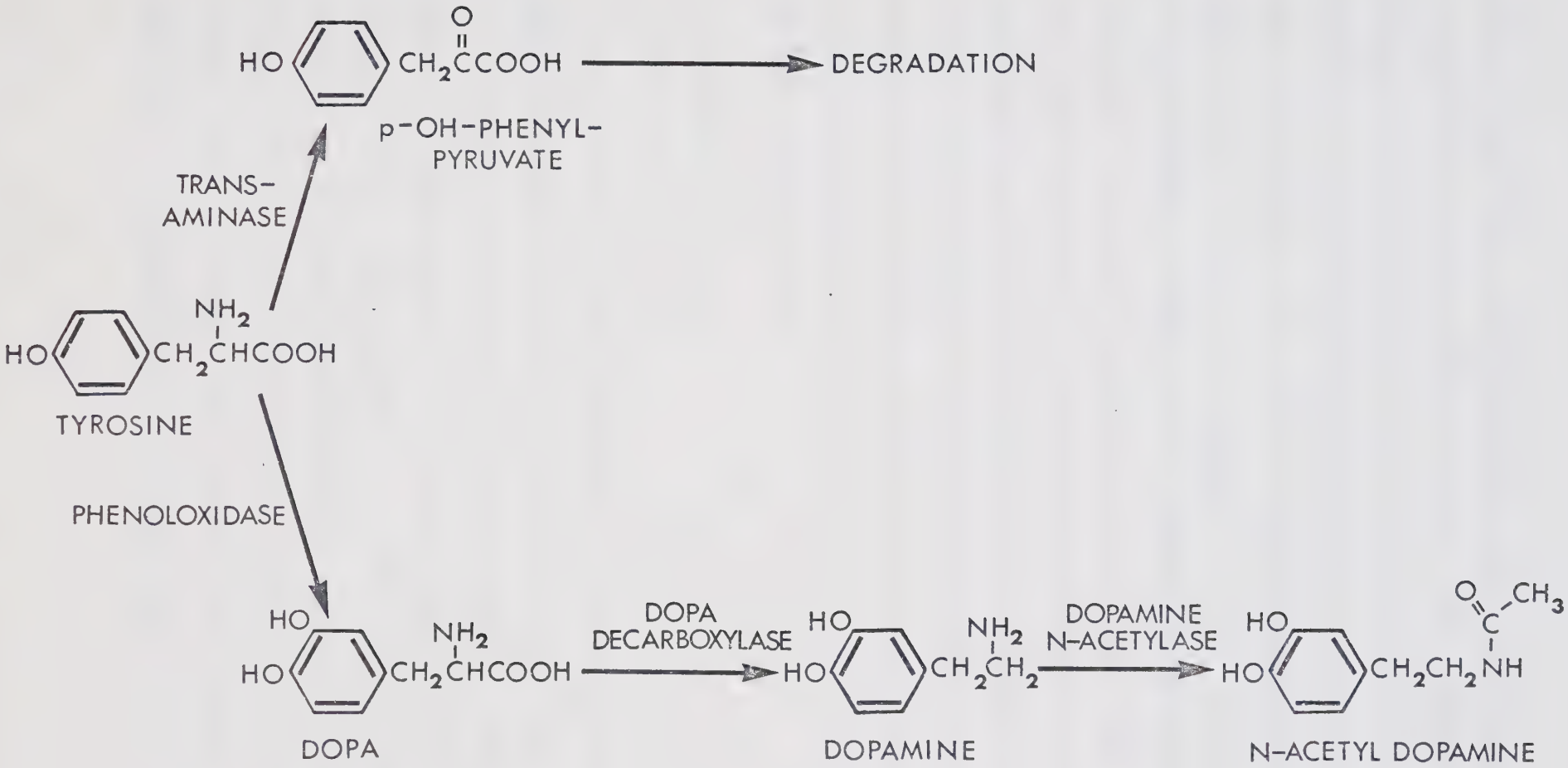


Figure 1.1. The metabolism of tyrosine in the blowfly, *Calliphora erythrocephala*. (Karlson and Sekeris, 1964)

posterior part of the cuticle in ligated larvae can be induced by the injection of the molting hormone, ecdysone. This clearly indicates that the process of sclerotization is under the control of this hormone. Furthermore, Karlson and Sekeris (1962) showed that ecdysone induced a substantial increase in the activity of dopa decarboxylase in the ligated animal. Although the biochemical basis of this observation and the mechanism of hormone action was pursued by Karlson and his co-workers for many years (Karlson and Sekeris, 1968; Sekeris et al., 1971), rather surprisingly, no attempt has been made to confirm their studies on enzyme induction in *Calliphora*, or to extend the observations to other systems. Therefore, one of the aims of this thesis is to extend Karlson's observation to another system and to develop an *in vitro* system of enzyme induction to circumvent the difficulties inherent in working *in vivo*.

(B) Ecdysone

(i) *Bioassay*

The biological activity of ecdysone is generally assayed by its capacity to cause the formation of a puparium in the larvae of certain flies. Fraenkel (1935) first reported that puparium formation could be induced in the posteria end of the ligated young larva of *Calliphora* by injection of blood from individuals that were ready to pupate. This experiment has led insect endocrinologists to develop a quantitative "*Calliphora* test" which has been used as a routine assay method for ecdysone (Becker and Plagge, 1939; Karlson, 1956). Recently, similar assays have been carried out in many other *Diptera* such as *Calliphora*

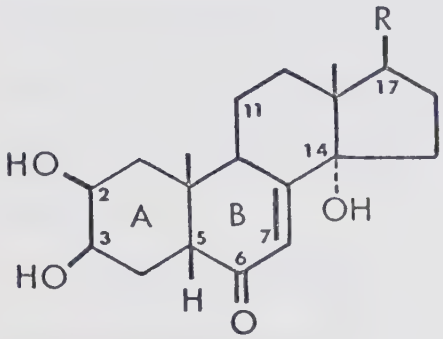
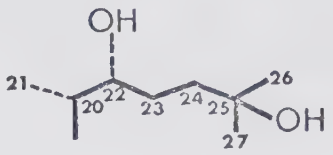
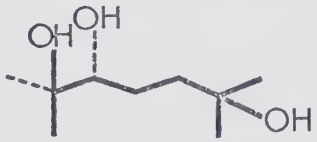
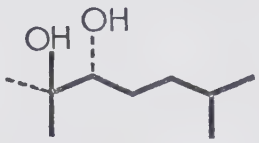
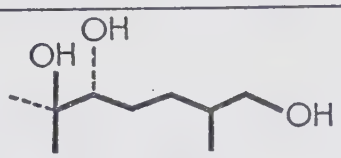
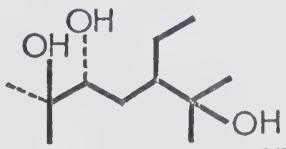
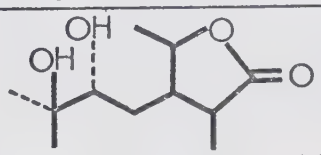
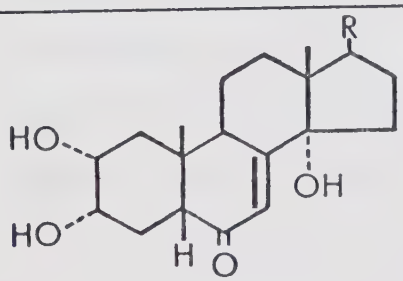
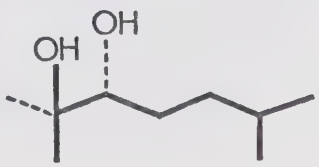
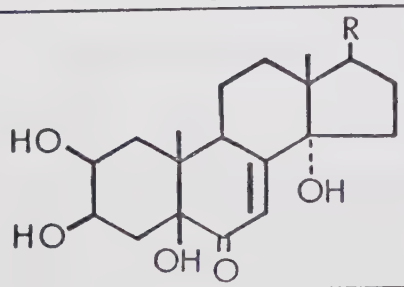
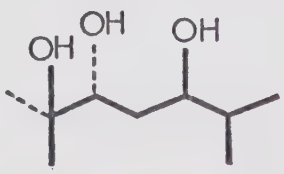
stygia (Thomson et al., 1970), *Sarcophaga peregrina* (Ohtaki et al., 1967), and *Musca domestica* (Staal, 1967; Adelung and Karlson, 1969), which gives greater sensitivity and precision. However, several factors such as *in vivo* metabolism of ecdysone, larval age at the time of ligation and the simultaneous action of an unidentified neurohormone have been found which complicate the abdomen test mentioned above (Fraenkel and Zdarek, 1970; King, 1972). In order to avoid these complications, Chihara et al. (1972) developed an *in vitro* assay method. In their method, the biological activity of an ecdysone preparation is assayed by its capacity to induce *in vitro* evagination of discs isolated from *Drosophila melanogaster*. This method is especially useful in assaying the biological activity of ecdysone analogues.

(ii) *Chemistry*

The development of the "*Calliphora* test" resulted in the successful isolation of a biologically active ecdysone from 500 kg of *Bombyx* silkworm pupae (Butenandt and Karlson, 1954). This compound was later designated as α-ecdysone by Karlson (Karlson, 1956; Karlson et al., 1963). By counter-current distribution separation methods, a second compound with molting hormone activity was isolated and designated as β-ecdysone by Hocks et al. (1967). Through extensive X-ray crystallographic and chemical analysis, the structure of α-ecdysone was determined to be a C_{27} sterol with exceptional polar character as a consequence of its multiple hydroxyl groups (Fig. 1.2), and β-ecdysone a 20-hydroxyecdysone (Huber and Hoppe, 1965; Hock and Weichert, 1966; Hoffmeister, 1966). The word ecdysterone is used to refer to β-ecdysone specifically, whereas

Figure 1.2. *Structures of ecdysones and some selected phytoecdysones with biological activity.*

(Wyatt, 1972)

RING SYSTEM	SIDE-CHAIN (R)	NAMES
		α-Ecdysone
		β-Ecdysone
		Ponasterone A
		Inokosterone
		Podecdysone A
		Cyasterone
		Ponasterone B
		Ponasterone C

ecdysone is often used loosely to designate α-ecdysone or β-ecdysone. Recent studies of different investigators have revealed that besides α- and β-ecdysone, there are many chemically related ecdysone analogues isolated from various plants which have high molting hormone activity (Nakanishi et al., 1966; Takemoto et al., 1967; Kaplanis et al., 1967). All these compounds are termed phytoecdysones (Fig. 1.2). The important structural features of the molecules that are responsible for its biological activity are: (a) the cis configuration in ring A and ring B; and (b) a C-22 hydroxyl group. The additional hydroxyl group in C-20 appears to increase molting hormone activity (see Wyatt, 1972, for review).

In *Bombyx*, *Calliphora*, or *Sarcophaga* larvae, α-acdysone is rapidly converted to β-ecdysone and β-ecdysone then undergoes further metabolism to biologically inactive polar metabolites (Ohtaki et al., 1968; Moriyama et al., 1970). Further studies indicated that the metabolism of ecdysone takes place either in the fat body or intestinal tract by enzymatic degradation (Shaaya, 1969). It appears that the degradation of ecdysones may involve more than one pathway, including side-chain scission to release a carboxylic acid (Galbraith et al., 1969), and glucoside formation (Heinrich and Hoffmeister, 1970).

(iii) *Biological effects*

Ecdysone-induced puparium formation in the *Diptera* which, as was mentioned above, provides the basis of the routine assay for ecdysone, is one of the most thoroughly investigated aspects of the hormone. As reported by Berreur and Fraenkel (1969), both the initial contraction of the larva into a puparial form and the cuticular sclerotization that

follows are dependent upon the presence of the hormone. In many of the *Diptera*, the hormone-induced hardening and darkening is brought about by phenolic tanning of the cuticular proteins. However, even in those members of the *Diptera*, where the hardening of the puparium occurs as a result of calcification, the process is under the control of ecdysone (Fraenkel and Hsiao, 1967).

A second effect of ecdysone is the induction of molting. Administration of ecdysone to the larval or pupal stages of an insect which is ready to molt but lacks endogenous hormone due to ligation or extirpation of the brain can induce molting promptly. Furthermore, Morohoshi and Iijima (1969) reported that ligated silkworm larval abdomens could be induced to molt immediately after the previous molt. Therefore it appears that ecdysone can induce molting at any stage, though less response to ecdysone is usually observed when the hormone is applied to an animal which is not ready to molt (see Wyatt, 1972, for review).

From the standpoint of the research undertaken in this thesis, perhaps the hormone-induced metamorphosis of the imaginal discs is the most significant of the developmental events under the control of the molting hormone. Imaginal discs are groups of cells of epidermal origin which, though apparently undifferentiated during larval growth, are destined to develop at metamorphosis into specialized adult structures. Studies on the transplantation of discs from larvae of *Drosophila* into the abdomens of adult flies revealed that the growth of discs is independent of the hormone, whereas metamorphosis of the discs requires the presence of ecdysone (Hadorn, 1966; Postlethwait and Schneiderman, 1968). Similar results have also been observed in the

wax moth, *Galleria mellonella* (Madhavan and Schneiderman, 1969). Many studies on imaginal discs cultured *in vitro*, including those on *Drosophila* (Fristrom et al., 1969; Chihara et al., 1972), *Galleria* (Oberlander, 1969, 1973), *Plodia* (Oberlander and Tomblin, 1972) and *Sarcophaga* (Ohmori and Ohtaki, 1973), indicated that the metamorphosis of the discs is dependent upon the presence of ecdysone. Most impressive are the recent experiments of Mandaron (1970, 1971, 1973), which demonstrated that discs isolated from *Drosophila* could be induced to undergo virtually complete development, including bristle formation and the deposition of adult cuticle. From the results presented above, it is clear that the imaginal discs are one of the true target tissues for ecdysone and their metamorphosis is dependent upon the presence of the hormone.

In addition to the morphological manifestations of hormone activity described above, ecdysone stimulates macromolecular syntheses, especially those of RNA and protein. Rapid stimulation of RNA and protein synthesis in several larval and pupal tissues of the silkmoth *(Hyalophora cecropia)* have been observed after injection of ecdysone (Berry et al., 1967; Wyatt, 1968; Sahota and Mansingh, 1970). *In vitro* studies on the effects of α-ecdysone on the synthesis of macromolecules revealed that the presence of α-ecdysone in cell nuclei isolated from epidermal cells or fat body cells of *Calliphora* strongly stimulates the incorporation of ^{14}C-uracil into RNA (Sekeris et al., 1965; Congote et al., 1969). The induction of RNA and protein synthesis in cultured discs isolated from *Drosophila* (Fristrom et al., 1969; Raikow and Fristrom, 1971; Fristrom, 1972) and *Sarcophaga* (Ohmori and Ohtaki, 1973) has also been investigated. As revealed by Fristrom (1972), the syntheses of rRNA and general protein

were elevated several-fold in the presence of ecdysterone. However, attempts to detect the induction of new species of RNA and protein in the cultured *Drosophila* discs by employing DNA-RNA hybridization techniques and SDS disc gel electrophoresis were unsuccessful.

(C) Rationale and Objectives

In order to present the rationale and objectives of this work, a general description of the process of sclerotization has been given. Most significantly, the enzymes involved in this process appear to be under the control of the molting hormone, ecdysone. The main objectives of this work are: *(a)* to characterize dopa decarboxylase, a key enzyme involved in the process of sclerotization, with respect to (i) its biochemical properties, (ii) its activity during development, and (iii) its tissue localization; *(b)* to confirm the hormone inducibility of dopa decarboxylase *in vivo*; *(c)* to establish *in vitro* culturing conditions for imaginal wing discs for studies on the induction of dopa decarboxylase by ecdysone.

Chapter II

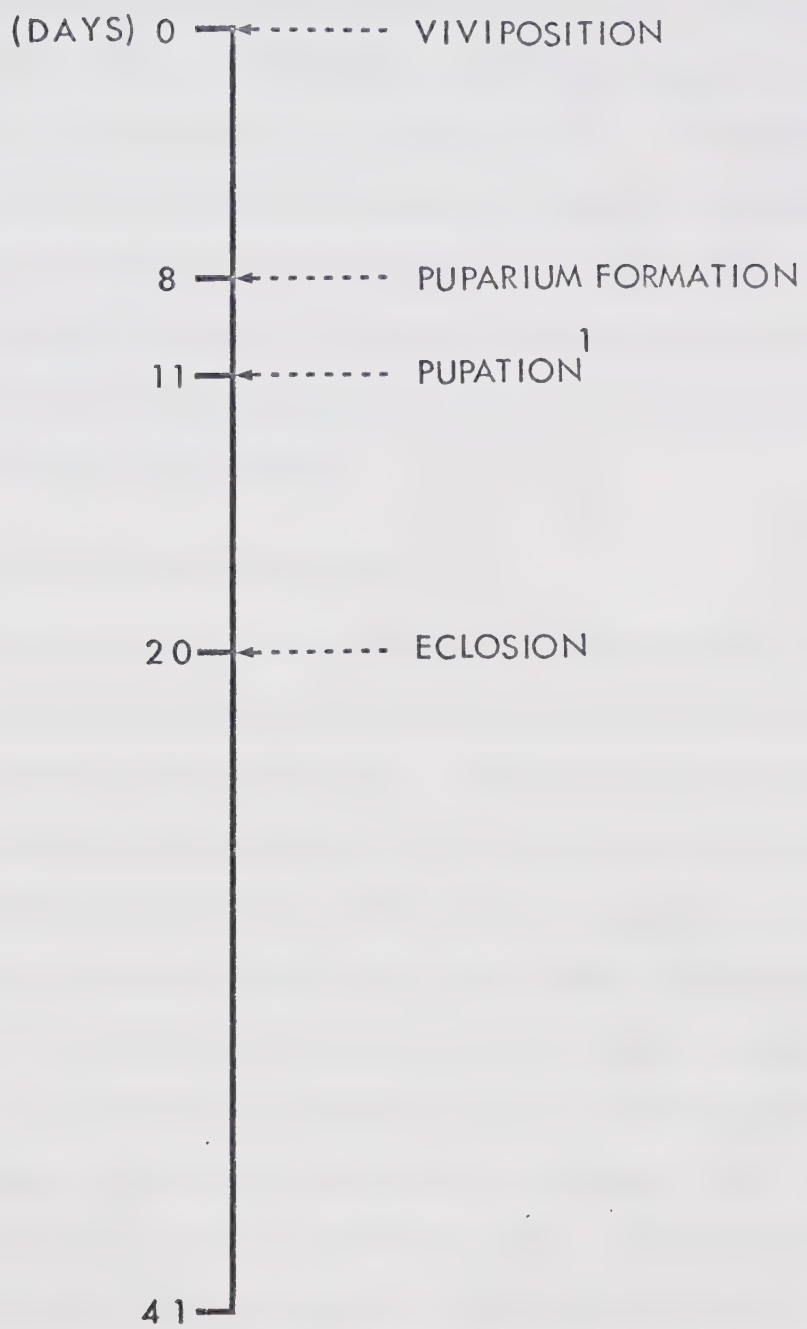
MATERIALS AND METHODS

(A) Maintenance of Flies

The fleshfly, *Sarcophaga bullata* Parker, was obtained from Dr. R. P. Bodnaryk. The animals were reared at room temperature (23 - 25°C), under continuous lighting and 20 - 30% relative humidity. Larvae were collected on raw ground beef in disposable petri dishes. Forty-eight hours later, larvae were transferred to fresh raw ground beef in plastic bags and then placed in a polyethylene container with dry paper towels. Under these conditions, larvae require 6-1/2 to 7 days for full growth. The mature third instar larvae crawl out of the meat, and wander for 24 to 36 hr on the dry paper before pupariation. Pupae, collected and kept in the dark in an incubator at 25°C, eclosed about 12 days after pupariation. Adult flies in a wooden cage were supplied with water and fed with a dry mixture of milk powder (Klim), glucose and yeast extract (Difco). Two weeks after adult eclosion, female flies are mature and ready to lay larvae. Adult flies normally lived for about 3 weeks. The life cycle of *Sarcophaga bullata* in our laboratory is summarized in Figure 2.1.

As reported by Ohtaki (1966), the secretion of the molting hormone, ecdysone, in *Sarcophaga peregrina* is prevented as long as mature larvae are stored in contact with water. For better synchrony of the culture in most of the experiments, mature, wandering larvae were collected and stored in contact with moist paper towels for 48 hr at room temperature

Figure 2.1. Life cycle of *Sarcophaga bullata* reared in laboratory.



[1]Zdarek and Fraenkel (1972).

and then transferred to dry conditions for further development. After this treatment, larvae formed puparia within 4 hr.

In order to simplify stock maintenance, an attempt was made to determine the feasibility of storing cultures in the cold. White puparia (0 - 2 hr old) were collected and incubated at 25°C for 2 days and then were transferred to a closed glass jar and kept at 4°C. At different time intervals, pupae were removed and checked for further development at 25°C. As shown in Table 2.1, the frequency of eclosion ranged from 20 - 50% over a period of 4 months. The adults which eclosed from cold treated pupae were still fertile, and this procedure was adopted as a routine for preserving the fly stocks.

(B) Assay of Dopa Decarboxylase Activity

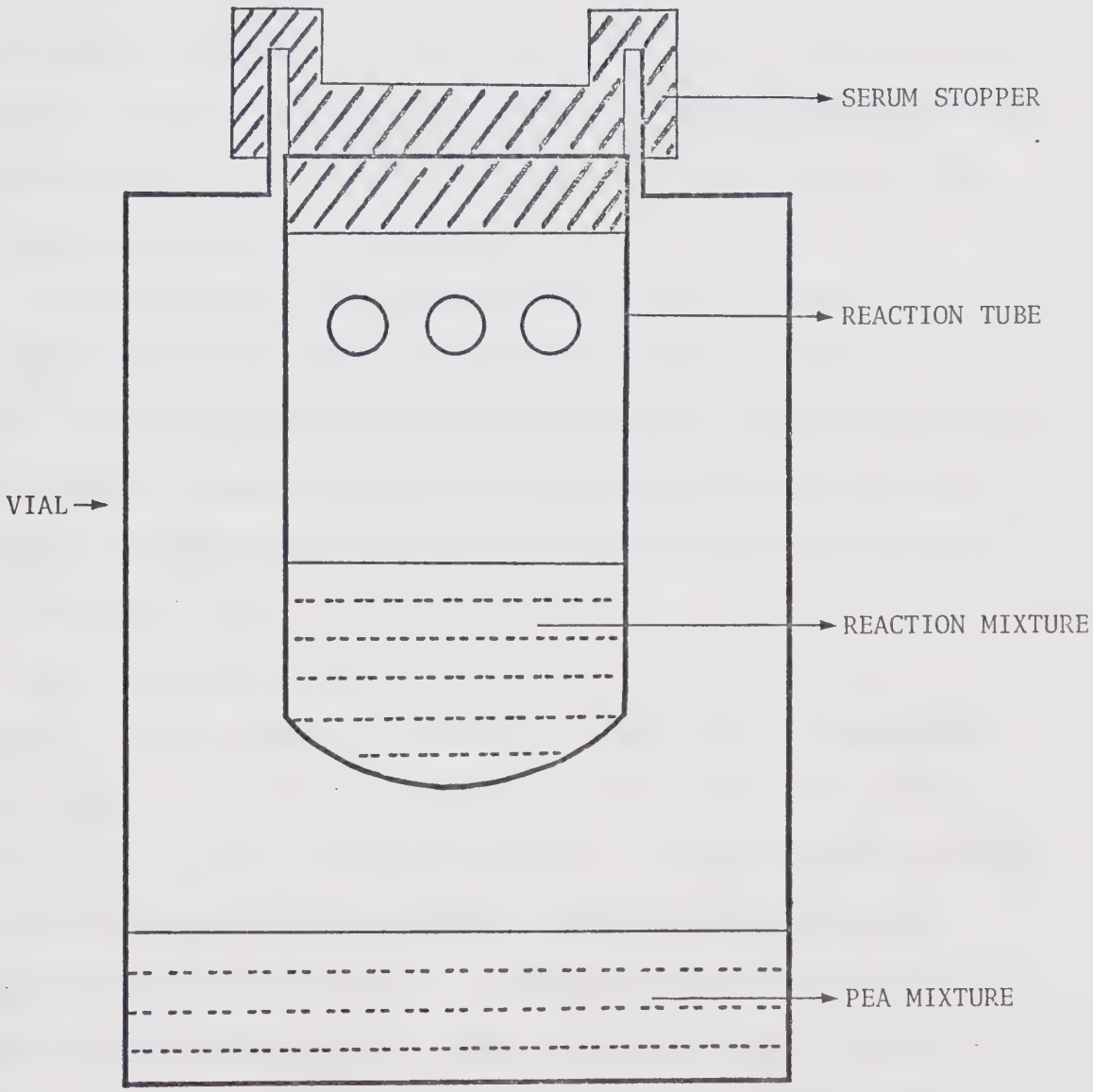
Dopa decarboxylase activity was measured by a slight modification of the carbon dioxide microdiffusion method of Lunan and Mitchell (1969), in which $^{14}CO_2$ is produced from 1-^{14}C-dopa. The reaction was carried out in a small polyallomer tube in which 6 - 8 holes had been punctured around the circumference near the top. The tube was suspended in a scintillation vial by a serum stopper (Fig. 2.2). The standard reaction mixture contained: Tris-HCl buffer (pH 7.5), 0.05 M; phenylthiourea (PTU), 2.4×10^{-3} M; pyridoxal-5'-phosphate, 1.2×10^{-4} M; DL-dopa-1-^{14}C (diluted with L-dopa to give specific activity 5.3 mC/mMole), 3.6×10^{-5} M; and enzyme extract to give a total volume of 0.5 ml. The reaction was initiated by injecting ^{14}C-dopa through the serum stopper with a Hamilton syringe, and after 20 min incubation at 37°C, it was terminated by adding 0.2 ml of 50% trichloroacetic acid (TCA). Diffusion of $^{14}CO_2$

Table 2.1. PERCENTAGE ECLOSION OF ADULTS STORED AT 4°C.

Time at 4°C (Days)	Time Required for Complete Pupal Development (Days)	% Eclosion
0	11	50
3	11	20
6	11	50
20	11	20
31	11	55
43	11	25
58	11	25
72	11	30
120	11	40

White puparia were collected for this experiment. The animals were incubated at 25°C for two days and then stored at 4°C. At different time intervals, 20 pupae were removed and incubated at 25°C for further development.

Figure 2.2. Diagrammatic representation of the reaction chamber used in dopa decarboxylase assay.



was allowed for a minimum of 2 hr at room temperature and the gas was trapped by 1 ml of a solution containing phenylethylamine (PEA)-methanol-toluene (1:1:2 by volume) in the bottom of the scintillation vial. Radioactivity was determined in a Packard Tricarb Liquid Scintillation Spectrometer by removing the reaction chamber and adding 10 ml of Bray's solution (Bray, 1960).

In our early experiments, $^{14}CO_2$ was trapped by KOH (10N) soaked in a small piece of Whatman #1 filter paper (1/2" x 1/2"). Later we found that the PEA mixture can trap $^{14}CO_2$ about 2-1/2 times more efficiently than KOH (Table 2.2). Therefore, the PEA mixture was used as a CO_2 trapping agent throughout this study.

It was found that ^{14}C-l-dopa decarboxylated spontaneously at an appreciable rate at alkaline pH, and therefore it was necessary to correct for spontaneous decarboxylation during the period of incubation. In the routine assays, sample values were corrected for the amount of spontaneous decarboxylation obtained in enzyme extracts inactivated at 60°C for 30 min (Figs. 2.3 a,b).

It was found that the optimum pH for the enzyme activity was between 7.5 to 8 in both 0.05 M Tris-HCl buffer and 0.05 M phosphate buffer (Figs. 2.4 2,b). The temperature of the water bath at which maximum enzyme activity was observed during a 20 min incubation was 40°C (Fig. 2.5). Since the enzyme activity decreases very sharply at temperatures higher than 40°C, an incubation temperature of 37°C was chosen for the standard assay. Under these conditions, the enzyme activity was directly proportional to the time of incubation (Fig. 2.6) and the amount of extract, over a considerable range (Fig. 2.7).

Enzyme extracts were prepared by grinding 2 well washed animals in

Table 2.2. COMPARISON OF CARBON DIOXIDE TRAPPING EFFICIENCY OF THE PEA MIXTURE[1] AND KOH (10N)

Trapping Agent	Apparent Enzyme Activity[2]	Efficiency (%)
PEA mixture	29.95	265
KOH (10N)	10.95	100

[1]Phenylethylamine : Methanol : Toluene = 1 : 1 : 2

[2]Apparent enzyme activity = *n*moles dopa decarboxylated/ml extract/20 min

A crude extract prepared from animals 12 hr after pupariation was used in this experiment.

Figure 2.3. *Heat inactivation of dopa decarboxylase.*

a) Heat denaturation of dopa decarboxylase activity. Extracts prepared from brown puparia were incubated at different temperatures for 15 min. Activity of dopa decarboxylase was determined by the standard procedure at 37°C.

b) Kinetics of denaturation of dopa decarboxylase at 65°C.

Extracts prepared from brown puparia were incubated at 65°C for various times. The activity of dopa decarboxylase was then determined by the standard procedure. Soluble protein was determined by the method of Lowry et al. (1951).

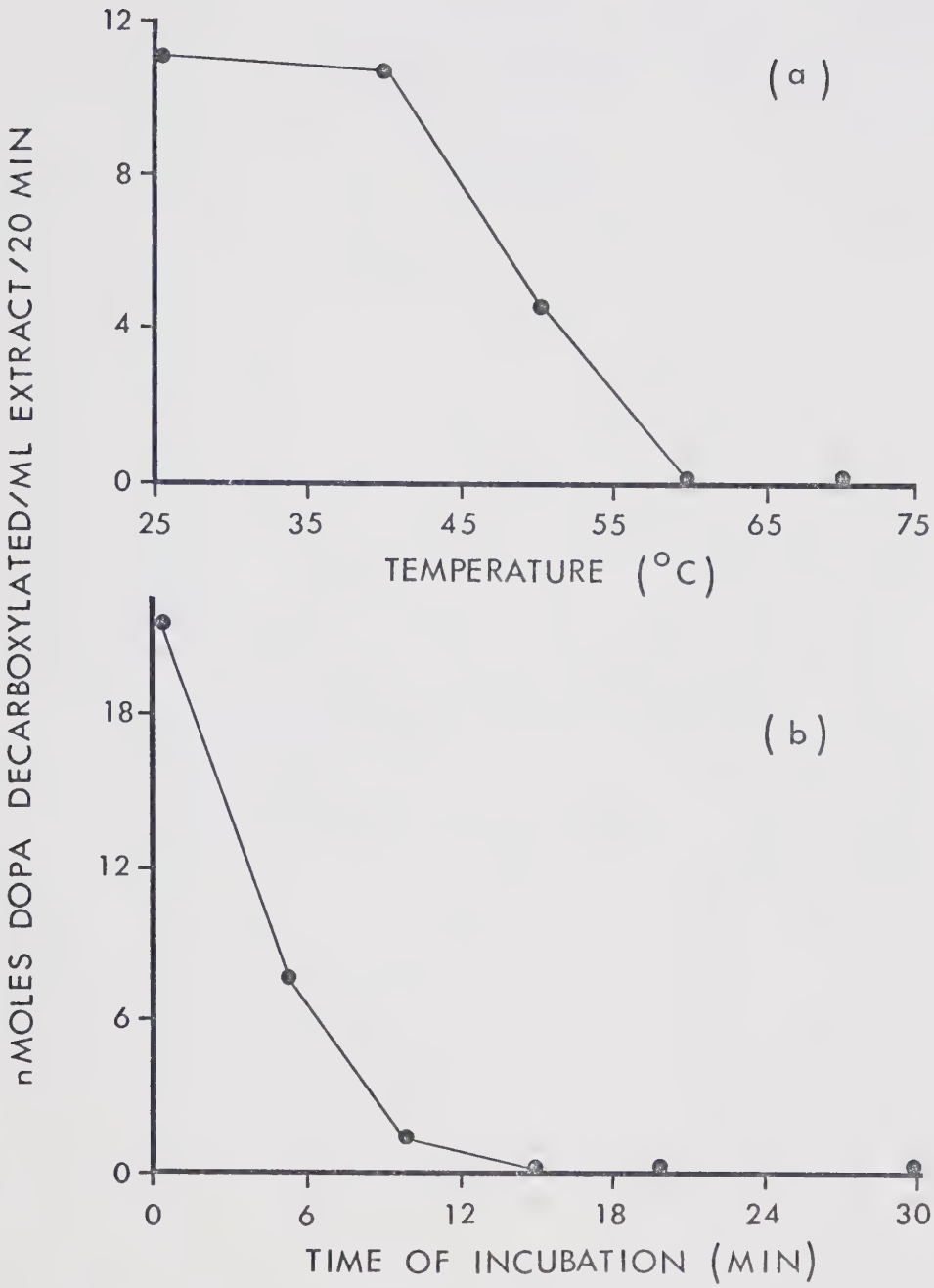

nMOLES DOPA DECARBOXYLATED/ML EXTRACT/20 MIN
12
8
4
0
25
35
45
55
65
75
TEMPERATURE (°C)
(a)
18
12
6
0
0
6
12
18
24
30
TIME OF INCUBATION (MIN)
(b)

Figure 2.4. *Activity of dopa decarboxylase as a function of pH.* Extracts were prepared from brown puparia in either Tris-HCl buffer (0.05 M) or sodium phosphate buffer (0.05 M). The reactions were carried out in *(a)* 0.05 M Tris-HCl or *(b)* 0.05 M sodium phosphate buffer at different pH's. Soluble protein was determined by the Lowry method.

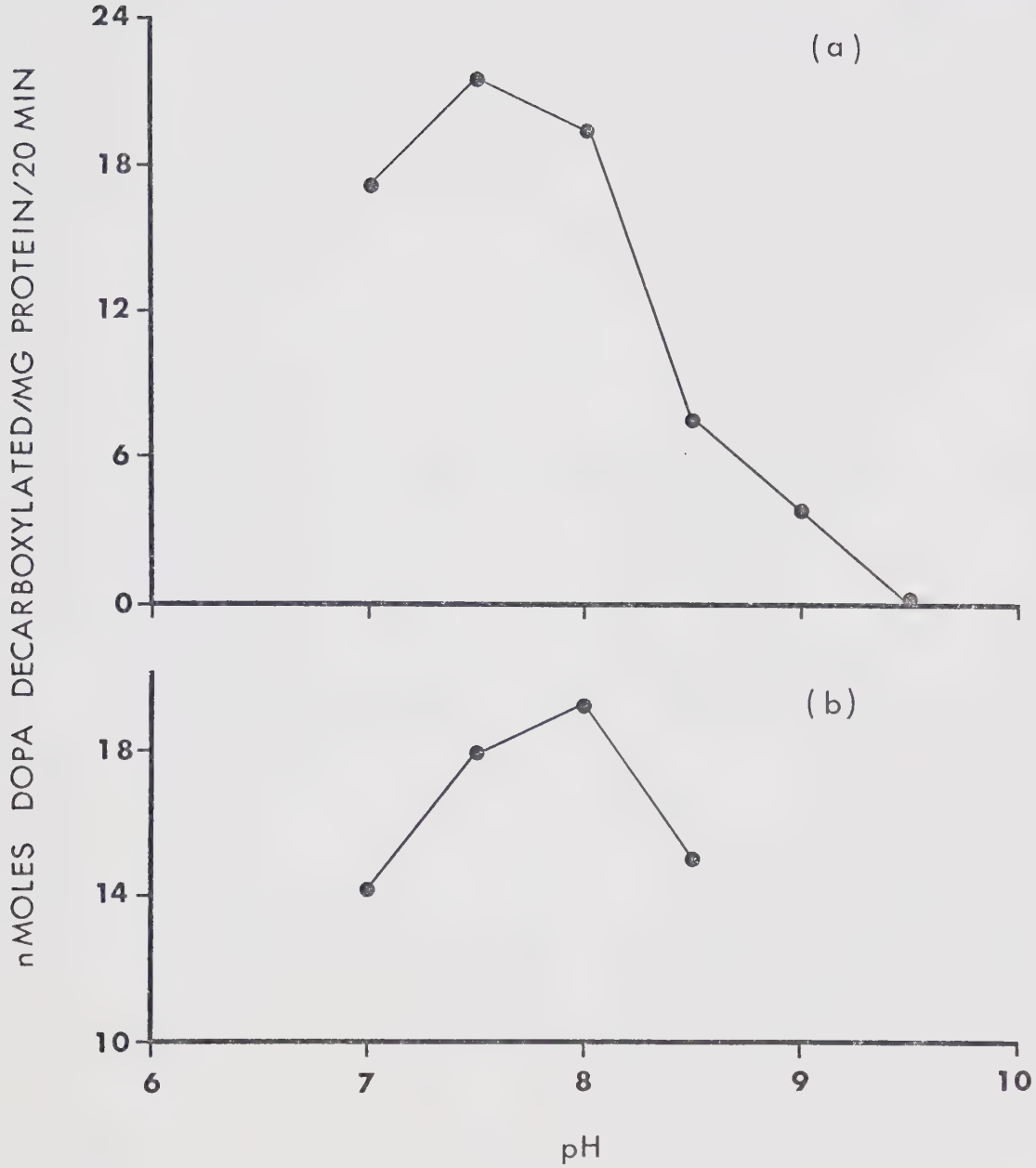

24
18
12
6
0
(a)
18
14
10
(b)
6
7
8
9
10
pH
n MOLES DOPA DECARBOXYLATED/MG PROTEIN/20 MIN

Figure 2.5. *Activity of dopa decarboxylase as a function of the assay temperature.*

Extracts were prepared from brown puparia and the reactions were carried out in 0.05 M Tris-HCl buffer (pH 7.5, adjusted at every temperature) for 20 min.

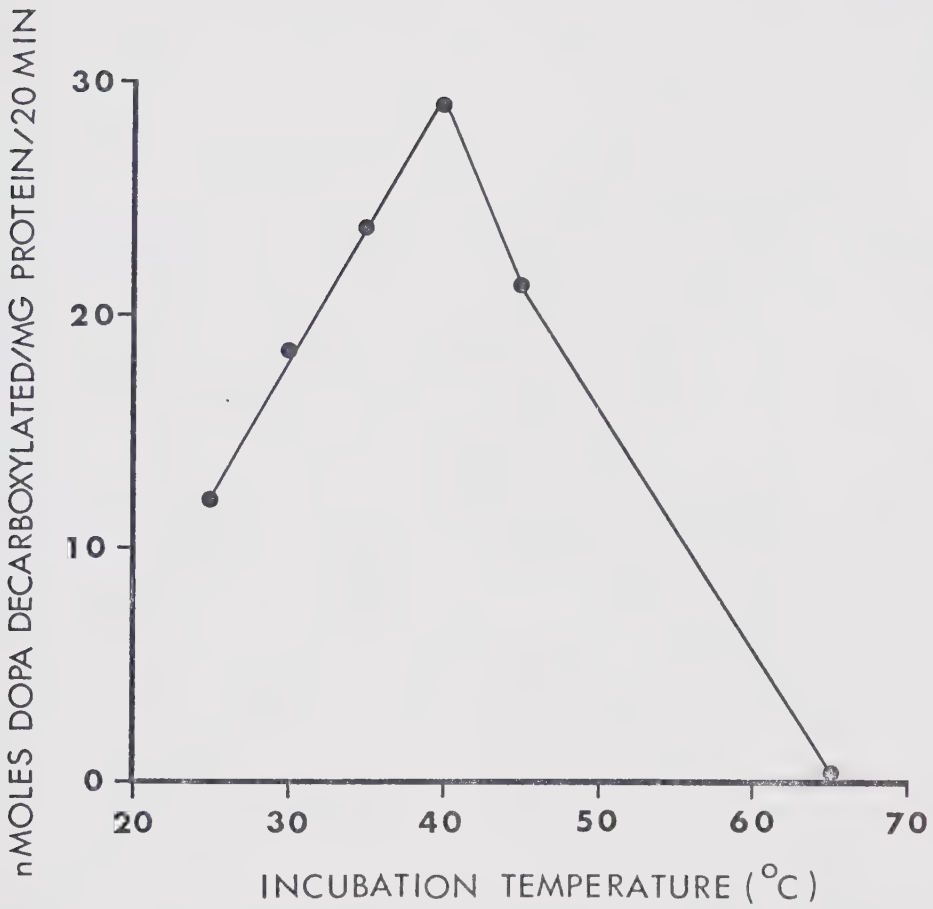

nMOLES DOPA DECARBOXYLATED/MG PROTEIN/20 MIN
30
20
10
0
20
30
40
50
60
70
INCUBATION TEMPERATURE (°C)

Figure 2.6. *Activity of dopa decarboxylase as a function of the incubation time.*

An extract was prepared by grinding 2 brown puparia in 1 ml of Tris-HCl buffer (0.05 M, pH 7.5). The supernatant of the centrifuged homogenate was diluted to 1/24 of the original concentration before using. The reaction was carried out in 0.05 M Tris-HCl buffer (pH 7.5) at 37^{o}C.

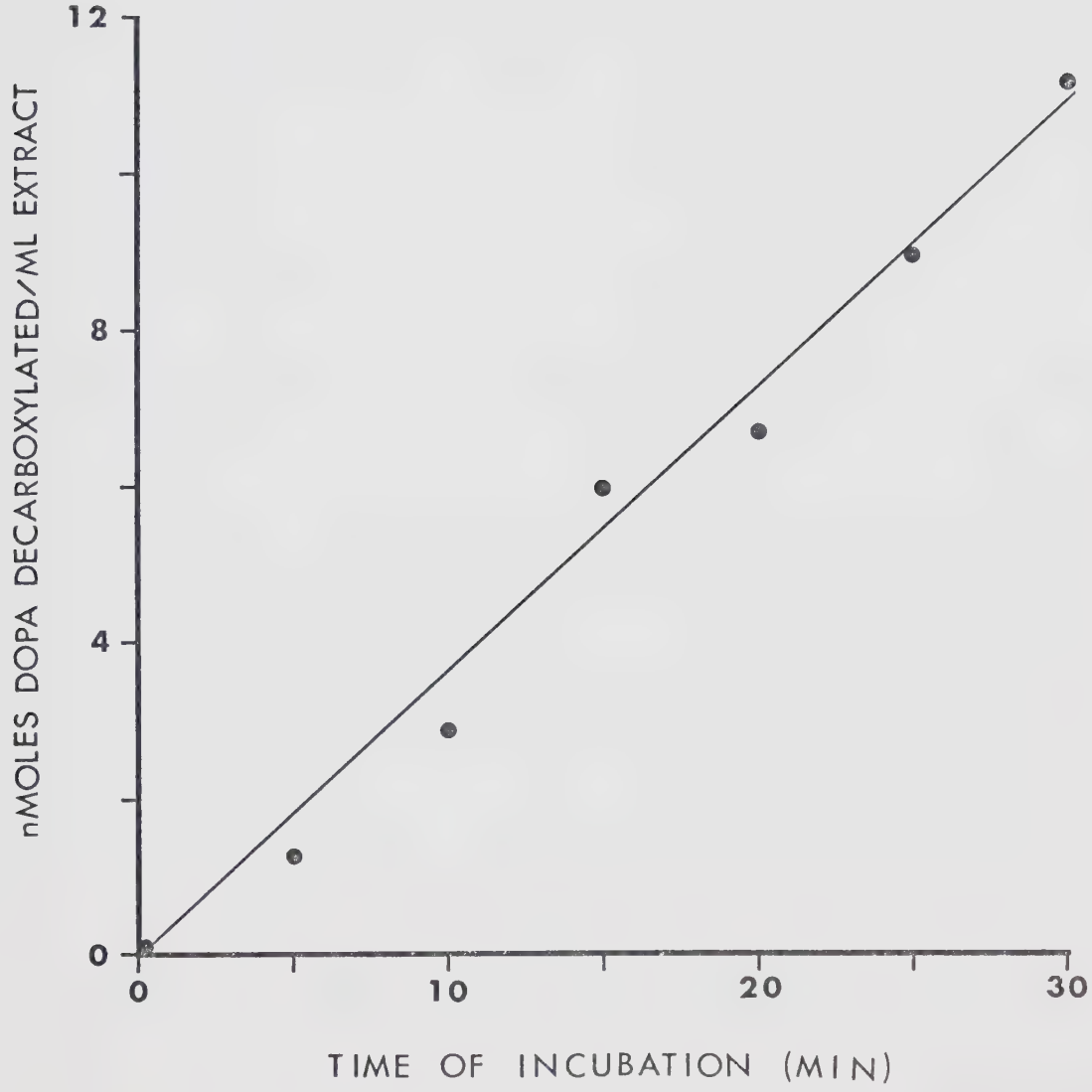
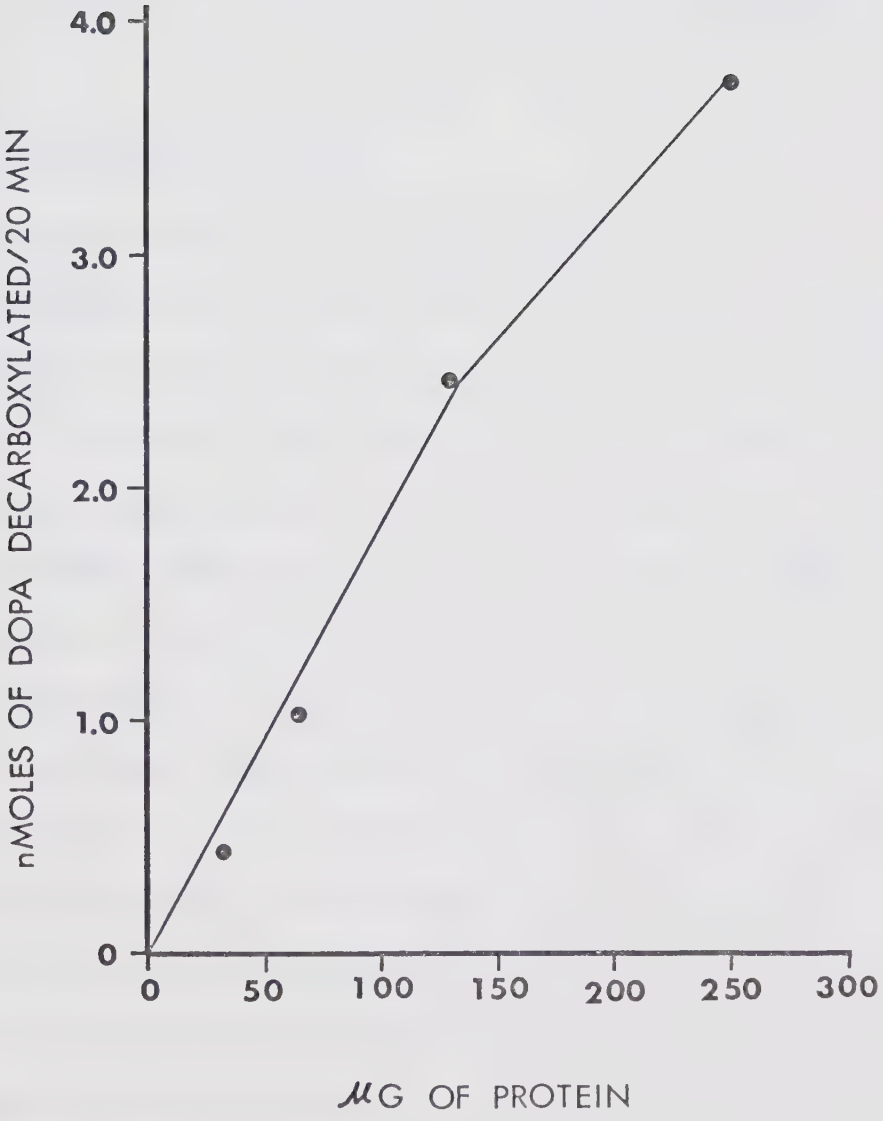

12
8
4
0
0
10
20
30
nMOLES DOPA DECARBOXYLATED/ML EXTRACT
TIME OF INCUBATION (MIN)

Figure 2.7. *Activity of dopa decarboxylase as a function of protein concentration.*

Extracts prepared from brown puparia were assayed for dopa decarboxylase activity in Tris-HCl buffer (0.05 M, pH 7.5) for 20 min at 37°C. Soluble protein was determined by the Lowry method.


4.0
3.0
2.0
1.0
0
nMOLES OF DOPA DECARBOXYLATED/20 MIN
0
50
100
150
200
250
300
μG OF PROTEIN

1 ml of 0.05 M Tris-HCl buffer (pH 7.5), containing PTU (2.4×10^{-3} M), in a glass homogenizer at 4°C. Debris was removed by centrifugation at 7700 g for 10 min in a Sorvall Refrigerated Centrifuge (RC2-B). In most of the cases, the centrifuged extracts were then passed through columns (0.9 x 10 cm) of Sephadex G-25 (Pharmacia Fine Chemicals) to remove small molecules. Proper dilutions were made on the dialyzed or undialyzed extracts to obtain 70 - 100 µg of soluble protein for each assay.

(C) Chromatographic Procedures

(i) *DEAE cellulose column chromatography*

Diethylaminoethyl Cellulose (DEAE, W. and R. Balston, England) was prepared by first stirring 100 gm of dry fiber with 1000 ml of 0.5 M NaOH for 1 hr at room temperature and the NaOH was removed by gentle suction on a Buchner funnel. The pH of the suspension was reduced to 8.0 by repeated rinses in distilled water and then the DEAE cellulose was resuspended in 1000 ml of 0.5 M HCl. The mixture was then stirred at room temperature and washed with glass distilled water to pH 4.0. Finally, the acidic DEAE cellulose was resuspended in 500 ml of 0.05 M Tris-HCl buffer (pH 7.5), stirred for 30 min and titrated with 6N KOH over a period of 6 hr at room temperature to a final pH of 7.5. A column, packed with the treated DEAE cellulose (1.2 x 14 cm) was equilibrated with 500 ml of 0.05 M Tris-HCl buffer (pH 7.5) at 4°C. Following sample application, the column was further washed with 20 ml of Tris-HCl buffer. Protein was then eluted with a linear ionic gradient containing 0.1 - 0.6 M NaCl in 0.05 M Tris-HCl buffer (pH 7.5).

The volume of the fractions collected was 3.0 ml.

(ii) *Sephadex column chromatography*

G-25 and G-200 Sephadex gels (Pharmacia Fine Chemicals, Montreal, Quebec) were prepared following the procedures described by Pharmacia Fine Chemicals. To hydrate the gels, 10 gm of G-25 were soaked in 500 ml 0.05 M Tris-HCl buffer (pH 7.5) for 3 hr at room temperature, or 10 gm of G-200 were soaked in 500 ml of Tris-HCl buffer for 3 days. After the gel was fully hydrated, the fine particles were removed by successively washing the gel with Tris-HCl buffer. Air bubbles trapped in the gel slurry during preparation were removed under vacuum. For dialysis purposes, G-25 was packed into a small column (0.9 x 10 cm) and equilibrated with 0.05 M Tris-HCl (pH 7.5) at 4°C. Columns could be used repeatedly if care was taken to wash them adequately after each run.

For gel-filtration column chromatography, the excess buffer used to swell G-200 gel was removed until a thick slurry remained. The slurry was then carefully pipetted into a column (1.2 x 55 cm) which was mounted vertically in the cold room. The column was connected to the buffer reservoir and the gel was packed under an operating pressure not exceeding 12 cm. The column was further equilibrated with 1000 ml of 0.05 M Tris-HCl buffer with or without mercaptoethanol (2.0×10^{-3} M). The void volume of the column was determined with 0.2% blue dextran-2000 (Pharmacia Fine Chemicals). In order to obtain good separation of the proteins, the amount of sample loaded on the column should be less than 2% of the total bed volume. Thus samples were always applied in volumes less than 0.5 ml. Following sample application, the column was eluted

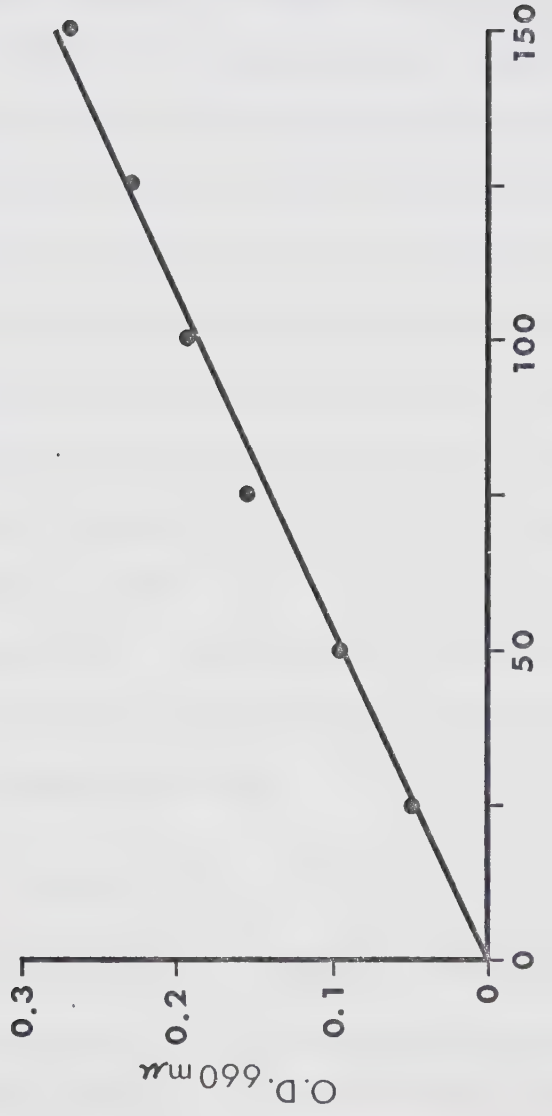
with 90 ml of Tris-HCl buffer (0.05 M, pH 7.5) with or without mercaptoethanol under an operating pressure of 12 cm. The volume of the fractions collected was 0.9 ml and the flow rate of the column was 6 ml/hr. After each run, the column was extensively washed with Tris-HCl buffer (0.05 M, pH 7.5).

(D) Determination of Protein Concentration

The concentration of protein in each extract was determined by the method described by Lowry et al. (1951). In this section, several difficulties encountered in applying the technique will be discussed. It was found that the PTU in the extract buffer reacted with the Folin reagent to give a false Lowry-positive reaction. This could be avoided by a prior dialysis procedure. Secondly, a slight false Lowry-positive reaction was observed when the Folin reagent was added to Tris-HCl buffer. Since the extracts were prepared in 0.05 M Tris-HCl buffer (pH 7.5), measurements of protein concentrations were corrected for blank values obtained by substituting the appropriate amount of Tris-HCl buffer for the extract.

The intensity of the blue color which develops in the procedure is a function of time. Therefore, throughout all the assays, we used one hour as the incubation time. All the reagents except the Folin reagent were prepared once every month. Each time when new reagents were prepared, a new standard protein concentration curve was obtained using bovine serum albumin as the standard. A typical standard curve is shown in Figure 2.8.

Figure 2.8. *Typical standard curve used for estimating protein concentration by the Lowry method.*
Bovine serum albumin, fraction V, was used as the standard protein.


O.D. 660 mμ
0.3
0.2
0.1
0
0
50
100
150
μG OF PROTEIN

(E) *In vitro* Culturing of Imaginal Wing Discs

Age synchronized mature larvae (48 hr on wet paper followed by 6 hr on dry paper) were stored at 4°C in the refrigerator for periods up to 10 days. Surface-sterilization was carried out by submerging the larvae in White's solution (White, 1963) for 40 min and then rinsing them three times in sterile distilled water. Imaginal wing discs were dissected from these larvae in Schneider's medium (Grand Island Biol. Co.) with sterile forceps, and rinsed at least three times in the same medium before being transferred to small sealed culture vessels (Bellco). In general, 10 discs were cultured in 2 ml of Schneider's medium supplemented with 10% heat inactivated fetal calf serum (Grand Island Biol. Co.), penicillin (100 units/ml) and streptomycin (100 µg/ml) at 25°C. This medium is called S medium throughout the text. Discs with injured membranes were discarded since membrane injury decreases the capability of further development *in vitro*.

For induction studies, 1 µg/ml (2×10^{-6} M) ecdysterone (prepared in 10% ethanol solution) was added to the medium, and this medium is called SE medium throughout the text.

(F) Chemicals and Hormones Used

The L-amino acid mixture-^{3}H(1 mC/ml 0.1N HCl), uridine-5-^{3}H (28.5 C/mMole), and DL-dopa-1-^{14}C (10.7 mC/mMole) were purchased from New England Nuclear (Boston, Mass.). α- cdysone was a gift from Dr. C.A. Henrick (Zoecon Corp.), and ecdysterone was purchased from Schwarz/Mann (Orangeburg, New York).

Chapter III

BIOCHEMICAL AND BIOLOGICAL CHARACTERIZATION OF DOPA DECARBOXYLASE IN SARCOPHAGA BULLATA

(A) Introduction

Dopa decarboxylase (3,4-dihydroxy-L-phenylalanine carboxy-lyase E. C. 4.1.1.26) catalyzes the conversion of dopa (3,4-dihydroxy-L-phenylalanine) to dopamine (3,4-dihydroxy-phenylethylamine) in the biochemical pathway leading to the biosynthesis of catecholamines from tyrosine in mammalian tissues (Molinoff and Axelrod, 1971). Several investigators have described the purification and characterization of this enzyme in different mammalian tissues (Fellman, 1950; Lovenberg et al., 1962; Christenson et al., 1970). Dopa decarboxylase isolated from mammalian tissues is specific for the L-isomers, but not specific for a particular aromatic L-amino acid. Because of its lack of substract specificity, the suggestion has been made to designate the enzyme as aromatic L-amino acid decarboxylase (Lovenberg et al., 1962; Christenson et al., 1970).

In higher *Diptera* as well as other insects, dopa decarboxylase is the key enzyme in the pathway of tyrosine metabolism leading to sclerotization, darkening and hardening of the larval cuticle during pupariation (Karlson and Sekeris, 1964; Lunan and Mitchell, 1969; Bodnaryk, 1970; Hodgetts and Konapka, 1973). Except for the work reported by Sekeris (1963) on the partial purification of dopa decarboxylase from *Calliphora erythrocephala*, very little is known about the biochemical properties of this enzyme.

In *Calliphora erythrocephala*, Shaaya and Sekeris (1965) observed that the profile of dopa decarboxylase activity during development was similar to the variation in the ecdysone titer. This suggested hormonal regulation of the enzyme, and further evidence supporting this was provided by the work of Karlson and Sekeris (1962, 1964, 1966). Rather surprisingly, no other workers attempted to confirm or extend these interesting studies. In this chapter certain of the biochemical and biological properties of dopa decarboxylase in *Sarcophaga* will be discussed.

(B) Results

(a) Biochemical characterization of dopa decarboxylase

(i) *Partial purification of dopa decarboxylase:*

In order to study the biochemical characteristics of dopa decarboxylase, a partially purified enzyme preparation was desired. The following steps were performed to obtain such a preparation.

(i.a) *Ammonium sulfate fractionation:* The first step in the purification scheme was ammonium sulfate fractionation. As shown in Table 3.1, about 80% of the total dopa decarboxylase activity was found in the fraction between 50 - 75% saturated ammonium sulfate. After this step of purification, the specific activity of the enzyme increased from 1.07 to 3.03 (i.e., 3-fold purification was obtained). As shown in Table 3.2, using a narrower concentration range of ammonium sulfate, a slightly higher purification was obtained.

(i.b) *Sephadex G-200 column chromatography:* The second step of purification was Sephadex G-200 chromatography. Figure 3.1 presents

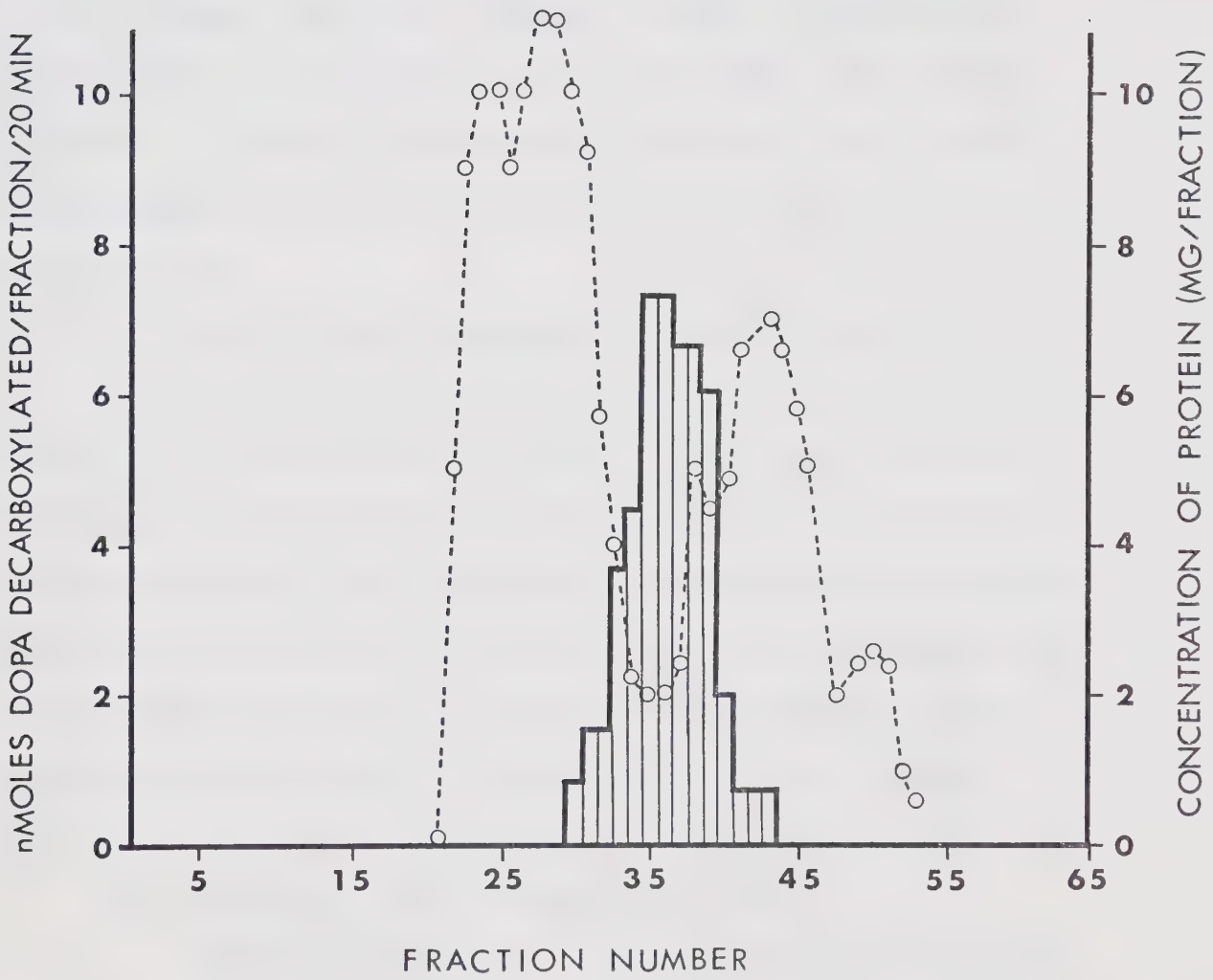
Table 3.1. AMMONIUM SULFATE FRACTIONATION

Saturation (%)	Activity of dopa decarboxylase (nmoles/fraction/20 min)	Total Activity (%)
0	219.8	100.0
0-50	33.3	14.3
50-65	99.5	42.6
65-75	84.8	36.3
75-85	16.1	6.9
85-100	0.2	0

A crude extract was prepared by grinding 12 brown puparia in 7 ml of 0.05 M Tris-HCl buffer (pH 7.5) with a glass tissue grinder at 4°C and the debris was removed first, by centrifugation at 7700 x g for 10 min and second by filtration through three layers of Kimwipes (Kimberly-Clark). A saturated ammonium sulfate solution was prepared by dissolving 770 gm of crystalline ammonium sulfate in 1000 ml of 0.05 M Tris-HCl (pH 7.5) and the pH of the solution was brought up to pH 7.5 by titrating with concentrated NH_4OH. The indicated degree of saturation was obtained by the dropwise addition of the neutralized saturated ammonium sulfate solution at 4°C. The precipitate collected by centrifugation at 12,000 x g and resuspended in 0.05 M Tris-HCl (pH 7.5). Enzyme assays were carried out in 0.05 M Tris-HCl buffer (pH 7.5) under the standard conditions.

Figure 3.1. *Chromatographic profile of activity of dopa decarboxylase on Sephadex G-200.*

An extract prepared from animals 12 hr after pupariation and fractionated by ammonium sulfate (55-70% saturation) was used in this study. After loading the 0.5 ml sample on the column (1.2 x 55 cm) of Sephadex G-200, proteins were eluted with 0.05 M Tris-HCl buffer (pH 7.5) at 4°C. Fractions of 0.9 ml each were collected. Protein was measured by the Lowry method and the enzyme activity was measured under the standard conditions in 0.05 M Tris-HCl buffer (pH 7.5). (·-·-O-·-·-O-·-) protein concentration; (————————) dopa decarboxylase activity.


nMOLES DOPA DECARBOXYLATED/FRACTION/20 MIN
CONCENTRATION OF PROTEIN (MG/FRACTION)
FRACTION NUMBER
0
2
4
6
8
10
5
15
25
35
45
55
65

a typical chromatographic profile of dopa decarboxylase on a Sephadex G-200 column. The activity of the enzyme was eluted between fractions 30 to 43. The specific activity of the enzyme at this stage of purification was 3.91 (Table 3.2). By comparing the recovery of total protein after Sephadex G-200 to the amount of protein in the 55 - 70% ammonium sulfate fraction, a 5-fold increase in the specific activity should have been obtained (Table 3.2). However, as Table 3.2 shows, there was no increase in the specific activity at all after this step of purification. In order to determine the cause of the loss in enzyme activity during Sephadex G-200 chromatography, the following experiments were carried out.

(ii) *The co-factor requirements of dopa decarboxylase:*

In order to examine the possibility that the decrease in activity on G-200 resulted from the loss of a co-factor, experiments were designed to determine the co-factor requirements of the enzyme. As presented in Table 3.3, a Sephadex G-200 enzyme preparation requires pyridoxal phosphate as an obligatory co-factor. The only metallic ion which has been tested is Fe^{+++}. Although a 40% elevation in enzyme activity was observed when Fe^{+++} was present in the assay mixture (Table 3.4), Fe^{+++} does not appear to be an obligatory co-factor.

(iii) *Stability of dopa decarboxylase at 4°C:*

The final possibility for the loss of activity on G-200 that was investigated was the question of enzyme stability. Experiments were therefore carried out to determine the stability of dopa decarboxylase at 4°C after different steps of purification. Table 3.5 presents the results of this study. It was found that 43% of the total enzyme

Table 3.2. PURIFICATION OF DOPA DECARBOXYLASE FROM BROWN PUPARIA

Fraction	Total Protein (mg)	Total[1] Activity	Specific[2] Activity	Purification
Crude extract	421.4	452	1.07	0
55-70% $(NH_4)_2SO_4$ fractionation	96.0	388	4.04	4
Sephadex G-200 chromatography	19.8	77.6	3.91	3.9

[1]Total activity is defined as: *n*moles of dopa decarboxylated/20 min.

[2]Specific activity is defined as: *n*moles of dopa decarboxylated/ mg of protein/20 min.

Table 3.3. DOPA DECARBOXYLASE ACTIVITY AS A FUNCTION OF PYRIDOXAL PHOSPHATE CONCENTRATION

Concentration of Pyridoxal Phosphate (M)	Activity of Dopa Decarboxylase (*n*moles/ml Extract/20 min)
0	0
1.2×10^{-6}	1.75
1.2×10^{-5}	3.22
1.2×10^{-4}	3.76
1.2×10^{-3}	2.46
1.2×10^{-2}	0

An enzyme extract from animals 12 hr after pupariation, purified by ammonium sulfate fractionation and Sephadex G-200 chromatography as summarized in Table 3.2, was used in this study. Activity of dopa decarboxylase was determined under the standard conditions in 0.05 M Tris-HCl buffer (pH 7.5).

Table 3.4. DOPA DECARBOXYLASE ACTIVITY AS A FUNCTION OF FERRIC ION CONCENTRATION

Concentration of Ferric Ion (M)	Activity of Dopa Decarboxylase (*n*moles/ml Extract/20 min)
0	1.85
5.0×10^{-5}	2.57
1.0×10^{-4}	2.25
5.0×10^{-4}	1.93
1.0×10^{-3}	0.23
5.0×10^{-3}	0

An enzyme extract from animals 12 hr after pupariation, purified by ammonium sulfate fractionation and Sephadex G-200 chromatography as summarized in Table 3.2, was used in this study. Ferric ammonium sulfate was used as the Fe^{+++} source. Activity of dopa decarboxylase was assayed under the standard conditions in 0.05 M Tris-HCl buffer (pH 7.5).

Table 3.5. STABILITY OF DOPA DECARBOXYLASE AT 4°C

State of Purification	% Remaining Activity		
	0 Hour	26 Hours	47 Hours
Crude Extract	100	43.0	16.0
55-70% Ammonium Sulfate Fraction	100	8.5	2.0
Sephadex G-200 Column Eluate	100	67.0	51.7

A crude extract was prepared from animals 12 hr after pupariation in 0.05 M Tris-HCl buffer (pH 7.5), and the enzyme activity was measured under the standard conditions in Tris-HCl buffer (pH 7.5).

activity remained in crude extracts stored at 4°C for 26 hr, 8.5% in the ammonium sulfate fractionated fraction and 67% in Sephadex G-200 fraction (Table 3.5). Further decreases in enzyme activity occurred when the different enzyme preparations were kept at 4°C for another 21 hr. The enzyme is most unstable after ammonium sulfate fractionation and least unstable after Sephadex G-200 chromatography. Repeated freezing and thawing at least 5 times did not cause any appreciable decrease in enzyme activity.

Attempts were made to stabilize the enzyme activity in the ammonium sulfate fractionated preparation by using pyridoxal-5'-phosphate, PTU, mercaptoethanol or L-dopa. As presented in Table 3.6, all the compounds except L-dopa stabilized the enzyme activity. The optimum conditions for stabilizing the enzyme activity were obtained when both mercaptoethanol and pyridoxal-5'-phosphate were present. In this case about 37% of the activity remained after 24 hr.

(iv) *Estimation of enzyme molecular weight:*

The apparent molecular weight of dopa decarboxylase was determined by gel filtration on Sephadex G-200. A Sephadex G-200 column (1.2 x 55 cm) was calibrated with 500 - 4000 μg of each of the following standards: myoglobin (M.W. 1.7×10^4), ovalbumin (M.W. 4.5×10^4), bovine serum albumin (M.W. 6.7×10^4), alcohol dehydrogenase (horse liver) (M.W. 7.3×10^4) and bovine γ-globulin (M.W. 1.6×10^5). The partition coefficients of each standard as well as dopa decarboxylase were calculated by the relationship (Laurent and Killander, 1964) $K_{avg} = (V_e - V_o) / (V_t - V_o)$, where V_e is the elution volume of the particular protein, V_t is the total volume of the gel bed, and V_o is

Table 3.6. THE EFFECT OF VARIOUS AGENTS ON THE STABILITY OF DOPA DECARBOXYLASE AT 4°C

Agent	Concentration (M)	Dopa Decarboxylase Activity (*n*moles/ml Extract/20 min)		Relative Stabilization[1]
		0 Hour	24 Hours	
Control		76.1	7.1	1.00
Phenylthiourea	2.3×10^{-3}	82.4	25.0	3.56
Mercaptoethanol	2.0×10^{-3}	94.4	25.2	3.55
Pyridoxal Phosphate	1.2×10^{-3}	78.5	17.8	2.51
Mercaptoethanol + Pyridoxal Phosphate	2.0×10^{-3} + 1.2×10^{-3}	91.5	34.4	4.76
L-Dopa	1.8×10^{-5}	74.9	4.7	0.66

[1]Relative stabilization = $\dfrac{\frac{Act_{24\ hr}}{Act_{0\ hr}} \text{ for treated extract}}{\frac{Act_{24\ hr}}{Act_{0\ hr}} \text{ for control extract}}$

An extract prepared in 0.05 M Tris-HCl buffer and fractionated in ammonium sulfate (55 - 70%) was used in this study. The enzyme activity was measured under the standard conditions in 0.05 M Tris-HCl buffer (pH 7.5).

the void volume. Figure 3.2 presents the elution profiles of the protein standards on Sephadex G-200. When K_{avg} of each individual protein standard was plotted against its respective molecular weight on a semi log scale (Figure 3.3), a linear relationship was observed. From 3 separate runs on G-200, the apparent molecular weight of dopa decarboxylase was determined to be $5.1 \pm 0.3 \times 10^4$ (Figure 3.3).

(b) Biological characterization of dopa decarboyxlase

(i) *Activity of dopa decarboxylase during development:*

Experiments were conducted to measure the activity of dopa decarboxylase at various stages of larval, pupal and adult development and the results are presented in Figure 3.4. In the early third instar larva, no appreciable amount of dopa decarboxylase activity was observed. Enzyme activity increased sharply during puparium formation and decreased to an undetectable level 4 days later (peak I). Another large peak of enzyme activity appeared 24 hr after adult eclosion and decreased again 4 days later (peak III). In addition, a smaller but sharper peak of enzyme activity was detected 5-1/2 days after puparium formation (peak II).

(ii) *Distribution of dopa decarboxylase at puparium formation (peak I):*

In order to determine whether the enzyme activity observed in peak I was localized in the cuticular epidermal cells as suggested by Lunan and Mitchell (1969), animals 12 hr after the white puparium stage were dissected in 0.05 M Tris-HCl buffer (pH 7.5, with PTU 2.4×10^{-3} M). The various fractions were assayed for dopa decarboyxlase activity. Table 3.7 summarizes the enzyme activity observed in different tissues. The data suggest that about 70% of the total dopa decarboxylase activity

Figure 3.2. *Chromatographic profile of protein standards on Sephadex G-200.*

The size of the column was 1.2 x 55 cm.

(—●———●—) γ-globulin (4 mg);

(--○---○--) ovalbumin (4 mg);

(—▲———▲—) myoglobin (2 mg);

(——————) alcohol dehydrogenase (0.5 mg).

γ-globulin, ovalbumin and myoglobin were determined by absorption at 280 mμ, and ADH was assayed according to Ahmed (1964).

Proteins were eluted with 0.05 M Tris-HCl buffer (pH 7.5).

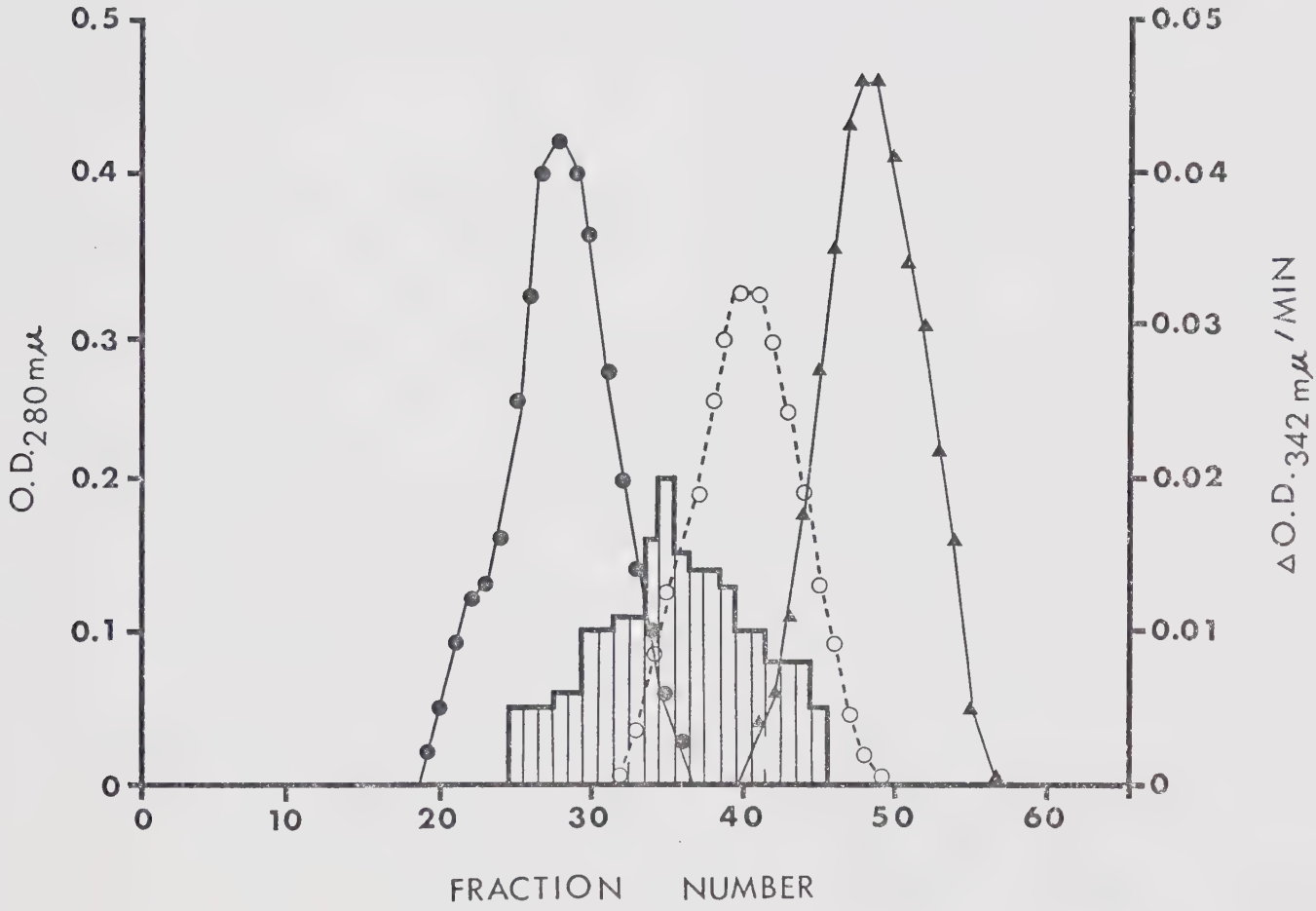

0.5
0.4
0.3
0.2
0.1
0
O.D.280mμ
0.05
0.04
0.03
0.02
0.01
0
ΔO.D.342 mμ /MIN
0
10
20
30
40
50
60
FRACTION NUMBER

Figure 3.3. *Correlation of the partition coefficient to the molecular weight.*

Partition coefficients of protein standards were calculated from the data obtained from Sephadex G-200 experiments according to the relationship:

$$K_{avg} = \frac{V_e - V_o}{V_t - V_o} .$$

The arrow indicates the K_{avg} of dopa decarboxylase activity. V_o was determined by blue dextran-2000. V_t was determined by calculating the volume of the gel from the height of the bed and the diameter of the column. The position of the centers of all peaks were determined by the relationship:

$$\text{Center of the peak} = \frac{\sum_i f_i \times c_i}{\sum_i f_i}$$

where the summation was taken over all the fractions (f_i) of the peak, in which enzyme activity or O.D.$_{280\ m\mu}$ (c_i) were found.

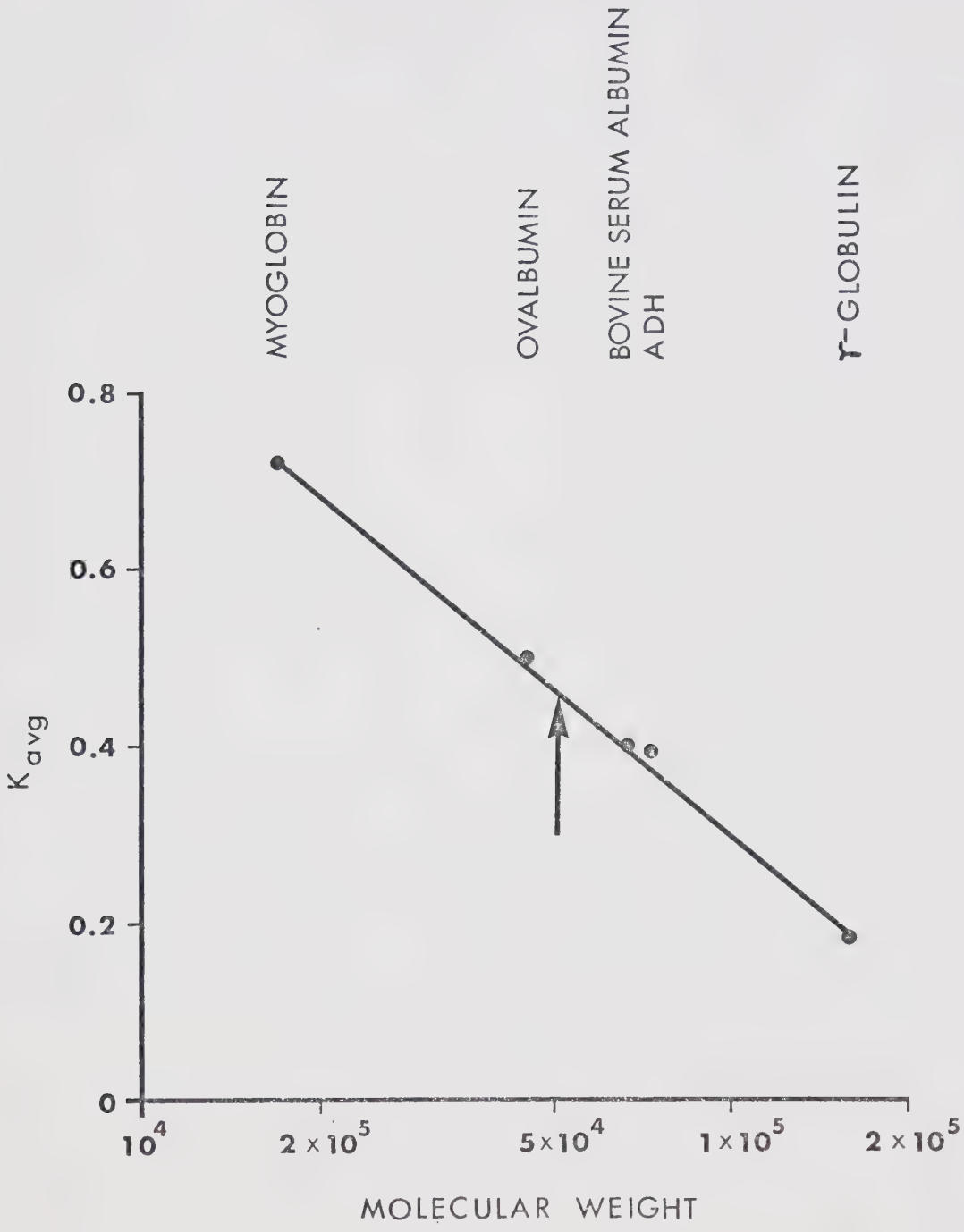

MYOGLOBIN
OVALBUMIN
BOVINE SERUM ALBUMIN
ADH
γ-GLOBULIN
0.8
0.6
0.4
0.2
0
K_{avg}
10^4
2×10^5
5×10^4
1×10^5
2×10^5
MOLECULAR WEIGHT

Figure 3.4. *Specific activity of dopa decarboxylase at different stages of development in* Sarcophaga bullata.
The age is expressed in days from the time of larval deposition. Each point is the mean of at least two determinations, and the S.E. of each point was less than 10%. The enzyme activity was measured under the standard conditions in 0.05 M Tris-HCl buffer (pH 7.5).

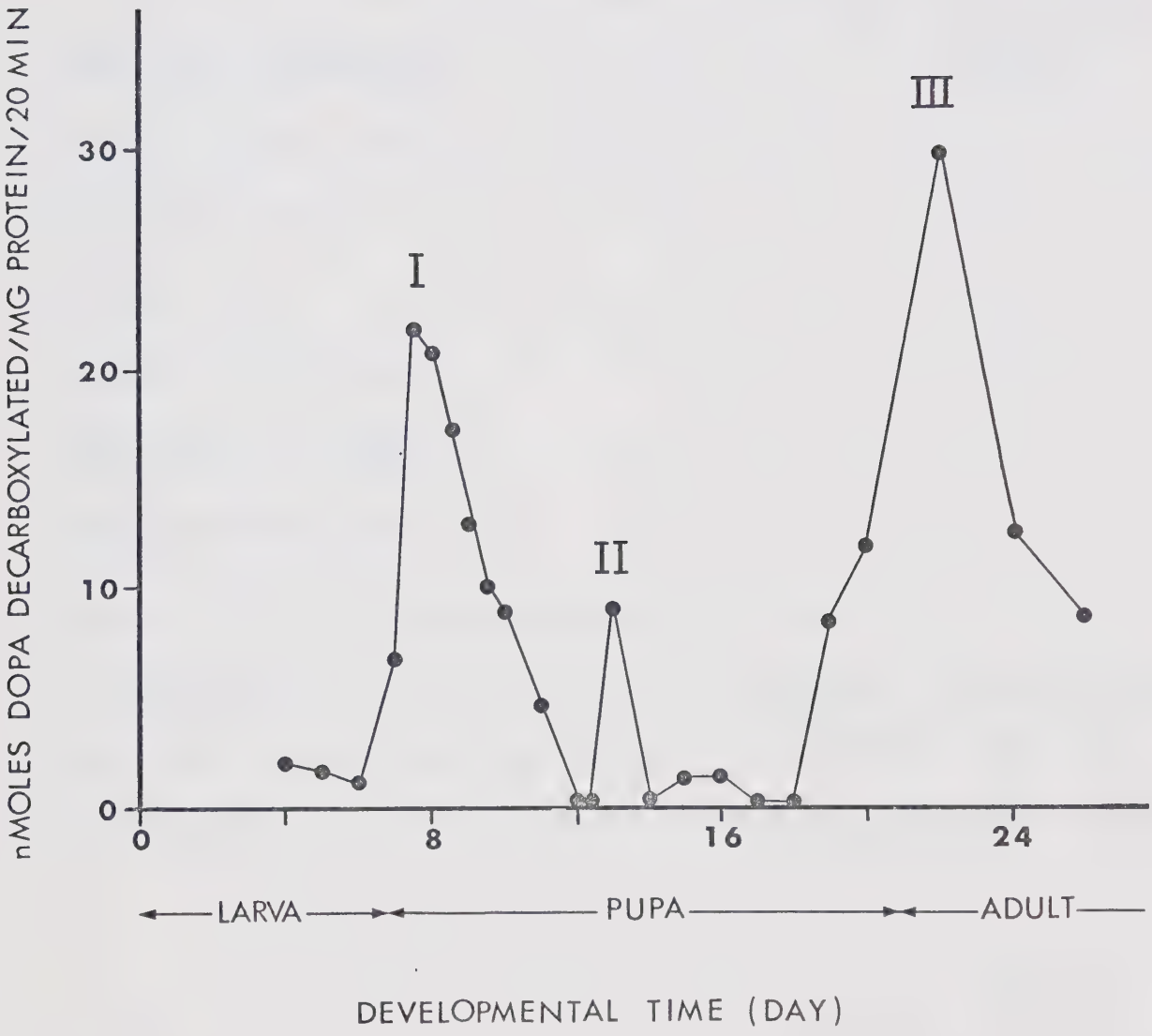

nMOLES DOPA DECARBOXYLATED/MG PROTEIN/20 MIN
30
20
10
0
I
II
III
0
8
16
24
LARVA
PUPA
ADULT
DEVELOPMENTAL TIME (DAY)

Table 3.7. DISTRIBUTION OF DOPA DECARBOXYLASE ACTIVITY IN *SARCOPHAGA BULLATA*, 12 HOURS AFTER PUPARIUM FORMATION

Fraction	Activity/Animal[1]	% Total Activity/Fraction
Blood Cells and Hemolymph	8.2	16.5
Cuticular Epidermal Cells	36.1	72.5
Muscles and Trachea	2.4	4.8
Fat Body	1.2	2.4
Imaginal Discs and Brain	1.9	3.8
Total Activity Recovered	49.8	100
Undissected Whole Animal	49.0	-

[1]Activity/animal = *n*moles of dopa decarboxylated/animal equivalent/20 min. Each point is the average of two experiments. The enzyme activity was measured under the standard conditions in 0.05 M Tris-HCl buffer (pH 7.5).

is in the larval epidermal cells lying just under the cuticle. Although a small fraction of the enzyme activity was found in blood cells, hemolymph, muscles, imaginal discs and the brain, it could well be due to contamination by cuticular epidermal cells.

(iii) *Forms of dopa decarboxylase in the organism:*

The decarboxylation product of this enzyme, dopamine, is thought to function as a neural transmitter in some organisms (Molinoff and Axelrod, 1971). Therefore we were interested to find out whether different forms of dopa decarboxylase were present in *Sarcophaga bullata* for sclerotization and neurological functions. Enzyme extracts prepared from whole animals about 12 hr after puparium formation or from isolated cuticular epidermal cells from animals of the same age, were chromatographed separately on DEAE cellulose. As shown in Figure 3.5 a and b, a single peak of dopa decarboxylase activity from these two different extracts was eluted off the column at the same ionic strength. Furthermore, dopa decarboxylase activity prepared from the heads of 10 days old adults, which were assumed to have virtually no cuticular dopa decarboxylase activity, showed the same chromatographic behavior on DEAE cellulose (Fig. 3.5 c).

(iv) *Induction of dopa decarboxylase activity* in vivo *by ecdysterone:*

In the results presented in Figure 3.4, peak I appeared at the stage when tanning of the larval cuticle is in progress. Bodnaryk (1971) reported that precocious tanning in young third instar larvae could be induced by the injection of exogenous ecdysterone, and this

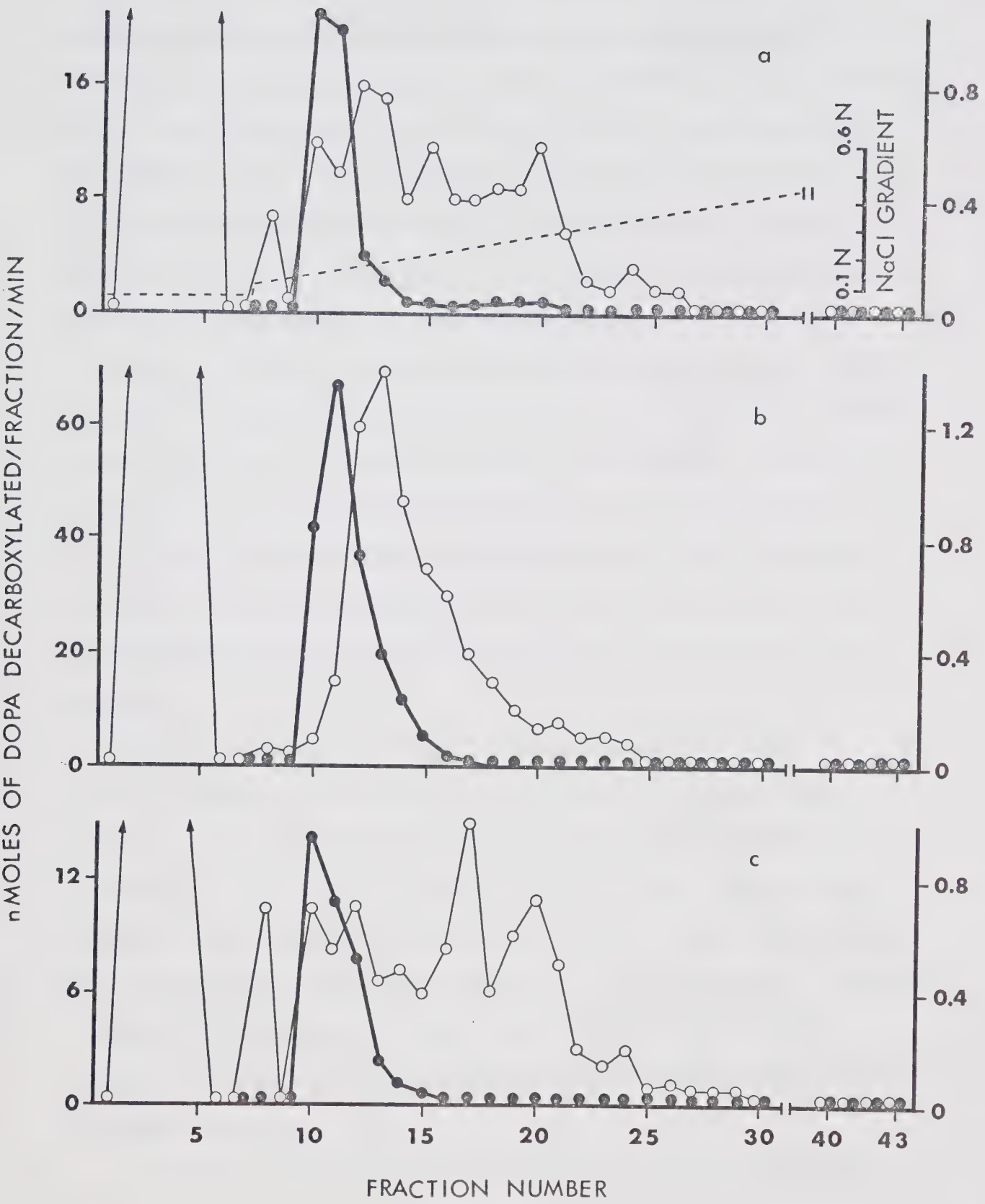
Figure 3.5. *Chromatographic profiles of dopa decarboxylase on DEAE cellulose.*

(a) The extract was prepared from whole animals, 12 hr after pupariation (brown puparia).

(b) The extract was prepared from cuticular epidermal cells isolated from brown puparia.

(c) The extract was prepared from the heads of 10 day old adults.

Crude extracts prepared from different sources were loaded to a 1.2 cm x 14 cm column of DEAE cellulose. After loading the 0.5 ml samples, the column was washed with 20 ml 0.05 M Tris-HCl buffer (pH 7.5). Proteins were then eluted with an ionic gradient consisting of 0.05 M Tris-HCl buffer (pH 7.5) and NaCl (0.1 to 0.6 M). Fractions of 3.0 ml each were collected. Protein was determined by absorbance at 280 mu (—o—o—), and the enzyme activity was measured under the standard conditions in 0.05 M Tris-HCl buffer (pH 7.5) (—●—●—). The ionic gradient of the eluting buffer (·····) is shown in (a).


a
b
c
16
8
0
60
40
20
0
12
6
0
0.8
0.4
0
1.2
0.8
0.4
0
0.8
0.4
0
0.6 N
0.1 N
NaCl GRADIENT
5
10
15
20
25
30
40
43
FRACTION NUMBER
nMOLES OF DOPA DECARBOXYLATED/FRACTION/MIN

induced tanning could be inhibited by DL-3-(3,4-dihydroxyphenyl)-2-hydrazino-2-methylpropionic acid (α-MDH), an inhibitor of dopa decarboxylase. These observations led us to speculate that dopa decarboxylase was induced in the latter experiments. To test this speculation, 120 hr larvae were taken from the meat, washed thoroughly and injected with 5 µl (20 µg) of ecdysterone in 10% methanol. Control animals were injected with 10% methanol. Animals were maintained at 4°C prior to injection to immobilize them and injection was performed using a Hamilton syringe. The animals were then maintained on wet paper at 25°C and the enzyme activity was measured at different time intervals. Precocious tanning was observed in the induced animals 24 hr after injection. A 4- to 5-fold increase in dopa decarboxylase activity was obtained 50 hr after ecdysterone injection (Fig. 3.6). The control animals showed no appreciable increase in dopa decarboxylase activity throughout the experiment.

Further experiments were carried out to locate the enzyme activity induced by ecdysterone in the precociously tanned animals. Animals, 30 hr after injection with ecdysterone were dissected in ice cold Tris-HCl (0.05 M, pH 7.5) with 2.4×10^{-3} M PTU. The cuticular epidermal cells were separated away from the rest of the tissues, and dopa decarboxylase activity was measured in the two fractions. As shown in Table 3.8, the activity of dopa decarboxylase in the cuticular epidermal cells of the induced animals is 10 times higher than that in the control animals.

In order to compare the dopa decarboxylase activity induced by

Figure 3.6. *Induction of dopa decarboxylase in young third instar larvae by ecdysterone.*

Young third instar larvae (120 hr) were injected with 20 µg/animal ecdysterone (in 10% methanol) and incubated at 25°C for various lengths of time. Control animals were injected with same amount of 10% methanol. The enzyme activity was determined under the standard conditions in 0.05M Tris-HCl buffer (pH 7.5) (—●——●—) dopa decarboxylase activity in induced animals; (···o··o··) dopa decarboxylase activity in control animals. Each point is the average of two experiments and the S.E. of each point is less than 5%.

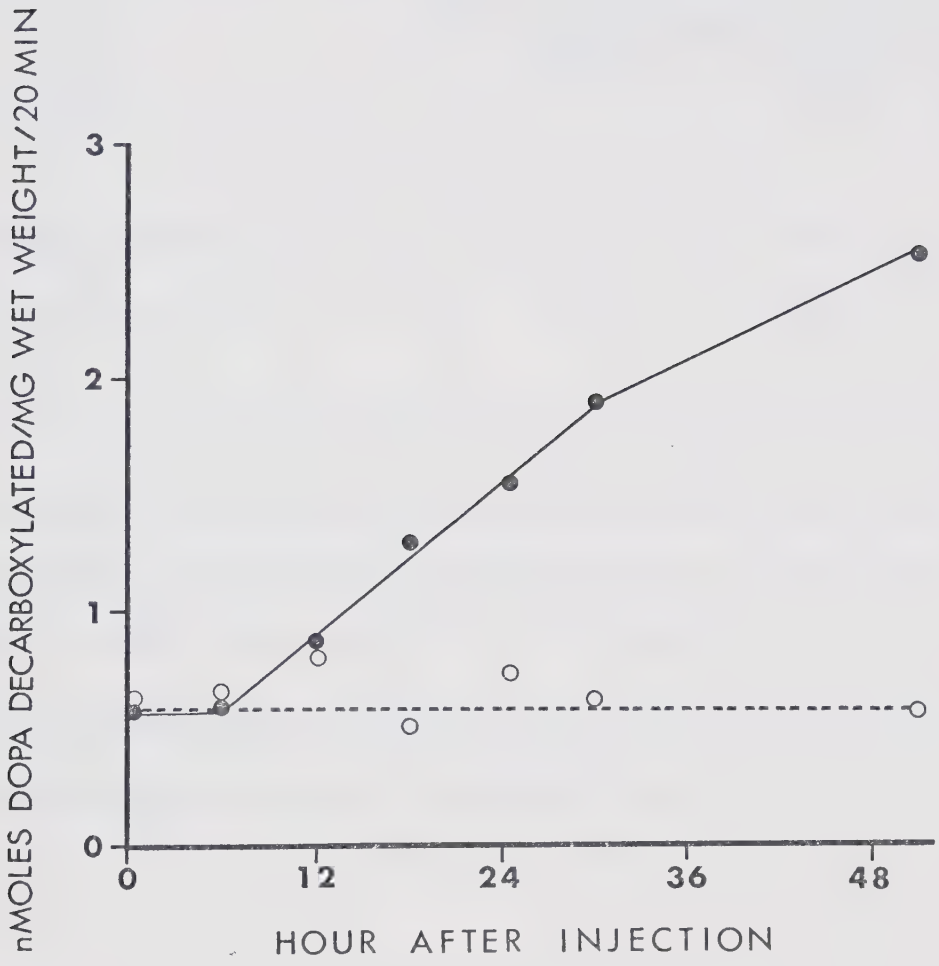

nMOLES DOPA DECARBOXYLATED/MG WET WEIGHT/20 MIN
3
2
1
0
0
12
24
36
48
HOUR AFTER INJECTION

Table 3.8. TISSUE DISTRIBUTION OF DOPA DECARBOXYLASE IN INDUCED AND CONTROL ANIMALS[1]

Tissue	Total Activity of Dopa Decarboxylase per Animal[2]	
	Control	Induced
Cuticular Epidermal	1.1	10.0
Remaining Tissues	8.1	6.1

[1]Young third instar larvae (120 hr) were injected with 20 µg/animal ecdysterone (in 10% methanol) and incubated at 25°C for 30 hr. Control animals were injected with 10% methanol.

[2]Total activity of dopa decarboxylase was expressed as: *n*moles dopa decarboxylated/animal equivalent/20 min.

ecdysterone in young third instar larva with that found in brown puparia, an extract was prepared from induced larvae and chromatographed on DEAE cellulose. A comparison of Figure 3.7 to 3.5 a or b shows that dopa decarboxylase from the prematurely induced animals was similar to that found at pupariation.

(C) Discussion

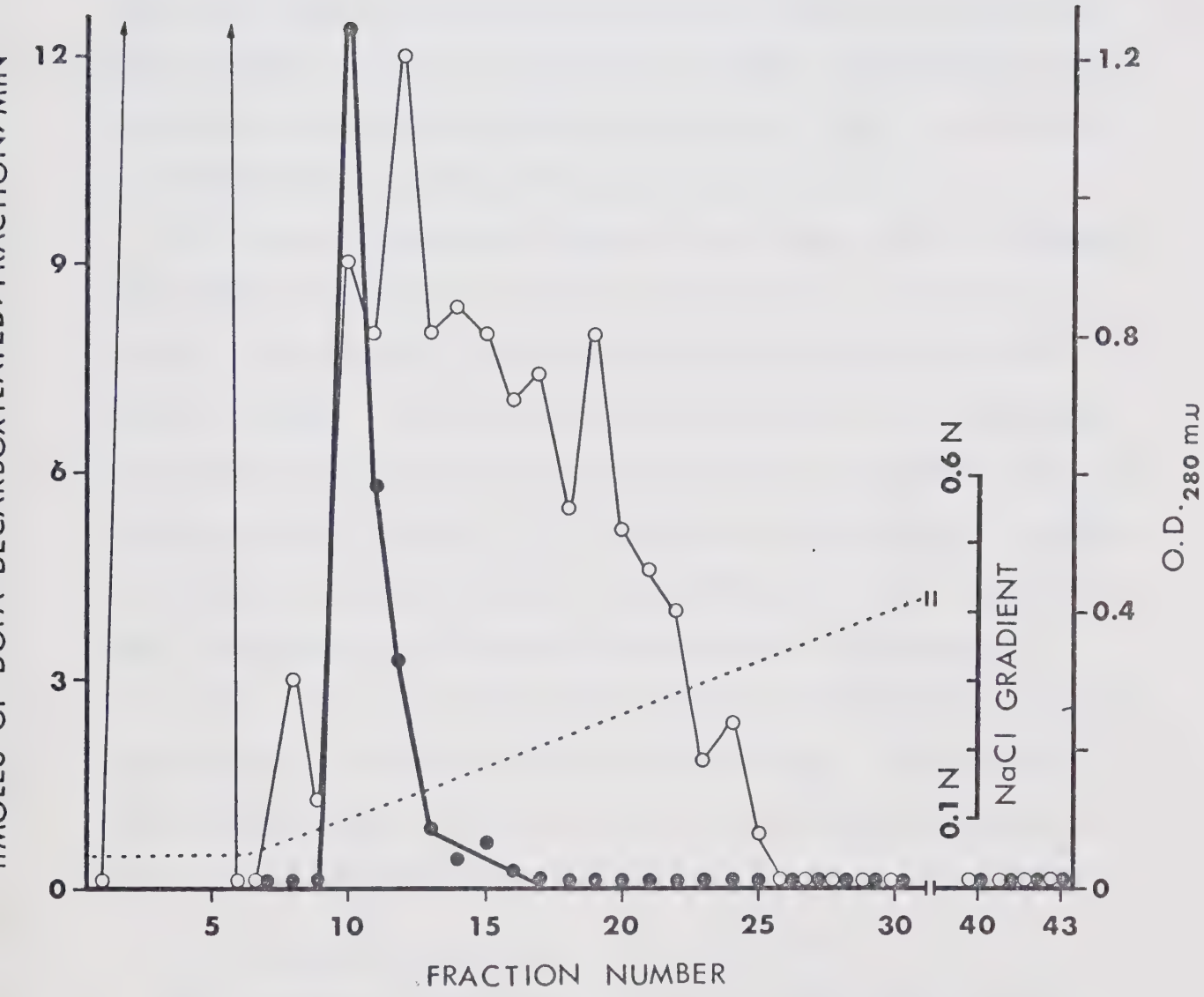
(a) *Biochemical properties of dopa decarboxylase*

Dialysed extracts of dopa decarboxylase show an absolute requirement for pyridoxal-5'-phosphate (Table 3.3). This observation is in accord with the results of Sekeris (1963) and Fellman (1950). In their studies on the aromatic L-amino acid decarboxylase in hog kidney, Christenson et al. (1970) claimed that ions such as Fe^{+++}, Fe^{++} and K^{+} had no effect on the rate of decarboxylation of dopa as well as other aromatic amino acids. In our studies, the rate of decarboxylation was elevated 40% above the control in the presence of Fe^{+++} (5.0×10^{-5} M) but no absolute requirement for Fe^{+++} was found. A similar observation has been reported by Sekeris (1963) in his study on dopa decarboxylase isolated from *Calliphora erythrocephala*.

From the studies on the stability of the enzyme at 4°C, it was found that an ammonium sulfate fractionated enzyme preparation is very unstable (Table 3.5). About 92% of the total enzyme activity is lost from a fractionated preparation after 26 hr. The reason for the lability following ammonium sulfate fractionation is unknown. However, it presents a problem in purifying the enzyme and probably contributed to our failure to achieve significant purification (Table 3.2). In order

Figure 3.7. *Chromatographic profile on DEAE cellulose of dopa decarboxylase from induced animals.*

Young third instar larvae (120 hr) were injected with 20 µg/animal ecdysterone (in 10% methanol) and incubated at 25°C for 50 hr. A crude extract was prepared from induced larvae and loaded on a 1.2 cm x 14 cm column of DEAE cellulose. The column was washed and eluted as described in the legend to Figure 3.5. Protein was determined by absorbance at 280 mu (—o—o—), and the enzyme activity was measured under the standard conditions in 0.05 M Tris-HCl buffer (pH 7.5) (—●—●—). The ionic gradient of the eluting buffer is indicated as (·····).


12
9
6
3
0
1.2
0.8
0.4
0
5
10
15
20
25
30
40
43
FRACTION NUMBER
0.6 N
0.1 N
NaCl GRADIENT
O.D.280 mμ

to overcome this problem, a rapid purification scheme must be employed.

An attempt to stabilize dopa decarboxylase was made using sulfhydryl compounds, pyridoxal phosphate and L-dopa. As indicated in Table 3.6, sulfhydryl compounds such as mercaptoethanol and phenylthiourea can stabilize the enzyme activity. It therefore appears that addition of these compounds to all buffers used during a purification procedure would be advisable. Sulfhydryl compounds also appear to activate the enzyme somewhat as can be seen from the zero time column of Table 3.5. This is consistent with observations on aromatic L-amino acid decarboxylase from mammalian systems (Christenson et al., 1970).

Gel filtration provides a rapid and simple method for the estimation of molecular weight. Studies by Andrews (1964, 1965) have shown that the elution volumes of proteins are largely determined by their molecular weights. The molecular weight of an enzyme can be determined by this method even when it is in a crude extract. Therefore, extensive purification of the protein is not required when this method is employed to estimate the molecular weight. As the data presented in Figure 3.3 shows, the apparent molecular weight determined by this method is $5.1 \pm 0.3 \times 10^4$. This is the first report on the apparent molecular weight of dopa decarboxylase isolated from an insect system although the molecular weight of aromatic L-amino acid decarboxylase from hog kidney has been reported to be 1.2×10^5 by Christenson et al. (1970).

(b) *Biological properties of dopa decarboxylase*

In our studies, three peaks of dopa decarboxylase activity were observed throughout the various stages of development of *Sarcophaga bullata* (Fig. 3.4). Peak I and peak III were found at puparium formation

and eclosion, and peak II was found at 5-1/2 days after puparium formation. Experiments on the tissue distribution of dopa decarboxylase at puparium formation revealed that at least 70% of the total enzyme activity is located in the cuticular epidermal cells (Table 3.7). This observation confirms the work of Lunan and Mitchell (1969) with *Drosophila melanogaster*. Peaks I and III are responsible for the sclerotization of the puparium and the adult cuticle respectively. Peak II is believed to be responsible for the sclerotization of pupal prothoracic spiracles (B. Heming, personal communication). Shaaya and Sekeris (1965), working with *Calliphora*, failed to observe this peak, either because of the insensitivity of their assay or the excessive time (24 hr) between their data points.

The homogeneity of the peaks of dopa decarboxylase activity observed on DEAE cellulose (Fig. 3.5) or Sephadex G-200 (Fig. 3.1) suggest that if the enzyme has a neurological function, this is not carried out by a distinct molecular species. This conclusion is supported by the fact that the enzyme from the heads of 10 day old adults has the same chromatographic properties as the enzyme located in the cuticular epidermal cells (Fig. 3.5 b, c). However, neither of these chromatographic methods has sufficient resolution to distinguish between two enzyme molecules with minor differences.

A superimposition of the activity profile of dopa decarboxylase in Figure 3.4 on the titer of the molting hormone, ecdysone, observed in various stages of development in *Calliphora erythrocephala* (Shaaya and Sekeris, 1965), indicates that peaks I and II fall under maxima in the ecdysone titer. The fact that the ecdysone titer is nearly zero

at eclosion raises the possibility that the appearance of dopa decarboxylase activity at this time (peak III) is not dependent on hormone action. Clearly though, the enzyme can be induced *in vivo* (Fig. 3.6), and its tissue specificity and chromatographic properties are similar to those of the enzyme normally found in brown puparia (Table 3.8 and Figs. 3.5 and 3.7). These results further confirm the observations on *Calliphora* (Karlson and Sekeris, 1962), in which an increase in dopa decarboxylase activity was obtained by the injection of ecdysone into the posterior portions of ligated larvae. Due to the complexity of the *in vivo* system, an alternative *in vitro* system was desirable to continue our studies on the nature of the hormonal induction of dopa decarboxylase. The development of this system is described in the following chapter.

Chapter IV

IN VITRO INDUCTION OF DOPA DECARBOXYLASE IN IMAGINAL WING DISCS BY ECDYSTERONE

(a) Introduction

Recent progress in the culturing of insect organs, tissues and cells now make both short- and long-term *in vitro* experiments possible. It has been reported that imaginal discs cultured in the presence of ecdysone can be induced to undergo considerable development, including the deposition of cuticle. Such studies now encompass work on *Drosophila* (Fristrom et al., 1969; Mandaron, 1970, 1971, 1973), *Galleria* (Oberlander, 1969; Oberlander et al., 1973), *Plodia* (Oberlander and Tomblin, 1972), and *Sarcophaga peregrina* (Ohmori and Ohtaki, 1973). The synthesis of RNA and protein are also stimulated when discs are cultured in the presence of molting hormones (Fristrom et al., 1969; Raikow and Fristrom, 1971; Ohmori and Ohtaki, 1973). This strongly suggests that imaginal discs are one of the target tissues for ecdysone. Recently, Wyatt et al. (1973) have shown that ornithine decarboxylase can be induced by ecdysterone in wing tissues of the silkmoth cultured *in vitro*. Therefore, we set out to determine whether or not cultured wing discs could be induced by ecdysone to produce dopa decarboxylase. The establishment of such a system would facilitate studies on the mechanisms of the hormonal induction of enzymes.

(B) Experimental Methods

(i) In vitro *synthesis of acid-insoluble RNA and protein*

Incorporation of tritiated precursors in total RNA and protein was measured by a modification of the method of Robb (Robb, 1969). In

each study, 20 - 30 intact discs were used and care was taken not to include discs damaged during dissection.

To measure the uptake of ^{3}H-uridine into total RNA, wing discs were cultured for various lengths of time in S or SE medium and then transferred to 1 ml of fresh S or SE medium supplemented with 10 µC ^{3}H-uridine (specific activity 28.5 C/mM). After an hour incubation at 25°C, incorporation was stopped by adding 0.5 ml of 10% TCA into the incubation mixture. Discs were homogenized in 2 ml of 10% ice cold TCA in a glass tissue grinder. After standing for 10 min at 4°C, the homogenate was filtered through a glass fiber filter (Whatman GF/C). The filter was washed with 20 ml of 10% cold TCA, 10 ml of 95% cold ethanol and 10 ml acetone, and then dried under an infrared lamp for 1 min. The dried filter was placed in 10 ml Bray's solution and the radioactivity was determined in Packard Tricarb Liquid Scintillation Spectrometer with 30% of counting efficiency.

In studying the incorporation of ^{3}H-amino acids into total protein, a similar procedure to that described above was used. However, incorporation was carried out in 1 ml of *Drosophila* phosphate buffer, pH 6.8 (Robb, 1969) supplemented with or without 2×10^{-6} M ecdysterone as required and with 10 µC of the ^{3}H-L-amino acid mixture.

Controls, in which the reaction was stopped at zero time, indicated that all acid soluble radioactivity was washed from the filters. The sterility of the disc cultures was checked by streaking the cultures on nutrient agar plates and incubating these at 37°C for 48 hr.

(ii) *Induction of dopa decarboxylase activity by ecdysterone*

Imaginal wing discs isolated from synchronized larvae were

pre-incubated in S medium for one hour. Groups of ten intact discs were transferred to small sealed culture dishes (3.0 x 1.2 cm, Bellco) containing 2 ml of SE medium and incubated at 25°C. At different times, the discs were removed from the medium and the activity of dopa decarboxylase was measured as described in Chapter II. For each time, a control was run by incubating 10 discs in S medium with the proper amount of 10% ethanol.

(C) Results

(i) *Effect of α-ecdysone and ecdysterone on the* in vitro *development of wing discs*

Schneider's medium, generally supplemented with serum, has been successfully used to maintain imaginal discs by several investigators (Schneider, 1964, 1967; Fristrom, 1969), and was the basis of our culture medium (see Chapter II). As Figure 4.1 shows, a considerable degree of hormone-induced development occurred when wing discs of *Sarcophaga* were cultured in SE medium. Discs cultured in S medium showed no appreciable morphological changes even after 96 hr (Fig. 4.1 a). The first observable morphological change in discs cultured in SE medium was the expansion of the discs to a somewhat round shape, which occurred 12 - 14 hr after exposure to the hormone (Fig. 4.1 b). At this stage of development, the peripodial membrane of the discs has become very thin and the folded wing structure can be seen through the membrane. Between 14 - 24 hr the peripodial membrane ruptured and the wing structure started to evert out of the sac (Fig. 4.1 c). After 40 hr of incubation in SE medium, the wing structure had everted completely (Fig. 4.1 d) and thereafter the presumptive wing continued to enlarge and elongate (Fig. 4.1 e).

Figure 4.1. *Development of imaginal wing discs cultured in SE medium.*

(a) Control disc. Control discs were cultured in S medium with 5 µl of 10% ethanol. The discs did not show any change in morphology throughout a 96 hr culturing period.

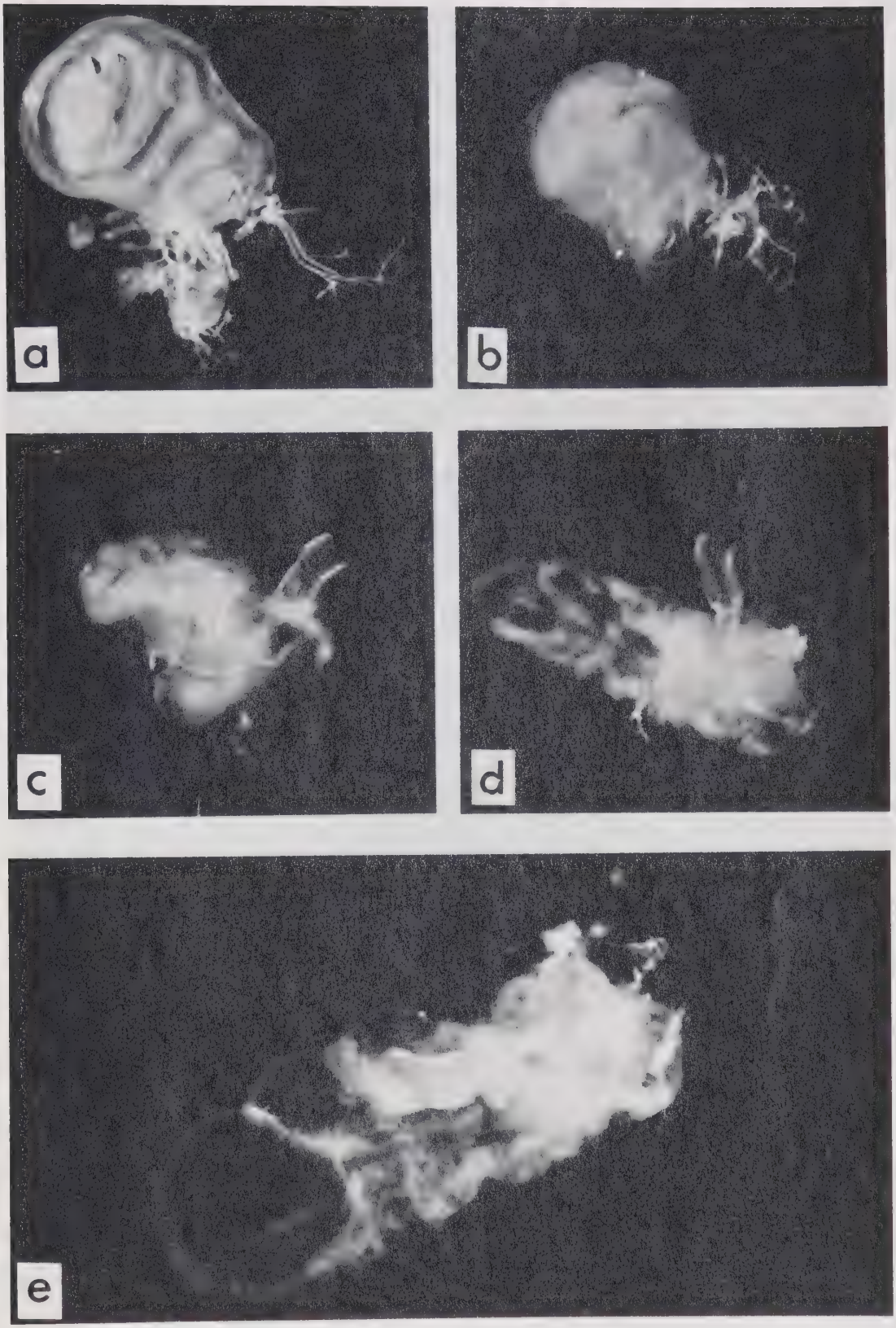
(b) Disc cultured 14 hr in SE medium. This stage represents the first appreciable morphological change. At this stage the peripodial membrane is very thin and no longer tightly adheres to the cells of the disc.

(c) Disc cultured 24 hr in SE medium. Peripodial membrane is partially ruptured, and the wing has begun to evert from the sac.

(d) Disc cultured 40 hr in SE medium. The presumptive wing has everted completely out of the sac.

(e) Disc cultured 64 hr in SE medium, considerable elongation has taken place since *(d)*.

Magnification: X 256.


a
b
c
d
e

The effect of α-ecdysone and ecdysterone on the development of wing discs cultured *in vitro* is summarized in Table 4.1. When discs were cultured in the presence of α-ecdysone (6×10^{-6} - 1×10^{-5} M), no significant development was observed even after 54 hr. Under the concentration of 2×10^{-5} M α-ecdysone, a slight development of the discs was observed after 54 hr (similar to Fig. 4.1 b). Complete evagination was observed in discs cultured in the presence of ecdysterone ranging from 2×10^{-7} to 1×10^{-5} M. Incomplete evagination was observed when high concentrations of ecydsterone (4×10^{-5} - 2×10^{-4} M) were used. Only slight morphological changes could be observed in discs cultured in the presence of 2×10^{-8} M ecdysterone. From these experiments, an ecdysterone concentration of 2×10^{-6} M was chosen as the optimum for further studies.

To determine the suitability of S medium for maintaining cellular integrity, discs cultured in S medium were transferred at various lengths of time to SE medium. As shown in Table 4.2, normal *in vitro* evagination was induced in discs that had been maintained in S medium for as long as 96 hr.

Table 4.3 presents the results of the effects of actinomycin D and cycloheximide on the *in vitro* evagination induced by ecdysterone. It was found that actinomycin D (1 µg/ml) or cycloheximide (10 µg/ml) inhibited *in vitro* evagination induced by ecdysterone when the inhibitors were added to the culture at zero hr. These concentrations also effectively inhibited macromolecular syntheses (Tables 4.4, 4.5).

Table 4.1. EFFECT OF α-ECDYSONE AND ECDYSTERONE ON DEVELOPMENT OF IMAGINAL WING DISCS

Hormones	Final Concentration (M)	Evagination[1]
α-ecdysone	6×10^{-6}	-
α-ecdysone	1×10^{-5}	-
α-ecdysone	2×10^{-5}	-[2]
ecdysterone	2×10^{-8}	-[3]
ecdysterone	2×10^{-7}	+
ecdysterone	1×10^{-6}	+
ecdysterone	2×10^{-6}	+
ecdysterone	5×10^{-6}	+
ecdysterone	1×10^{-5}	+
ecdysterone	4×10^{-5}	+[4]
ecdysterone	2×10^{-4}	+[4]

[1]Evagination of the discs was scored 30 - 40 hr after the addition of hormone, at which time under optimal conditions the discs appeared as shown in Figure 4.1 d.

[2]The discs had assumed the shape shown in Figure 4.1 b at 54 hr but no further development was observed.

[3]The discs had assumed the form shown in Figure 4.1 b after 24 hr of incubation but no further development was observed.

[4]Incomplete development resulting in irregular, distended discs.

Table 4.2. ABILITY OF IMAGINAL WING DISCS TO EVAGINATE FOLLOWING AN INCUBATION PERIOD IN S MEDIUM

Time in S Medium (hr)	Evagination
48	+
72	+
96	+

Discs were isolated from synchronized larvae and three groups of 5 discs were transferred to SE medium following a period of culturing in S medium.

Evagination was scored 30 - 40 hr after exposure to ecdysterone.

Table 4.3. EFFECT OF ACTINOMYCIN D AND CYCLOHEXIMIDE ON *IN VITRO* EVAGINATION INDUCED BY ECDYSTERONE

Inhibitor	Evagination
Control	+
Actinomycin D	-
Cycloheximide	-

Five discs, isolated from synchronized larvae, were cultured in SE medium. Actinomycin D (1 µg/ml) and cycloheximide (10 µg/ml) were added to the culture at 0 hr. Since actinomycin D is somewhat unstable, discs incubated in SE medium with actinomycin D were transferred to a fresh medium every 24 hr.

Evagination was scored 30 - 40 hr after induction.

(ii) *The ability of imaginal wing discs cultured in S medium to incorporate ^{3}H precursors into total RNA and protein*

One of the biochemical criteria for judging the viability of discs maintained *in vitro* is the ability of discs to synthesize total RNA and protein. Figure 4.2 indicates that discs cultured in S medium were able to incorporate ^{3}H-uridine into total RNA linearly for at least 72 hr. However, these data give no indication of the instantaneous rate of RNA synthesis. Instantaneous rates of RNA synthesis in discs cultured in S medium for different lengths of time were measured by the amount of incorporation of ^{3}H-uridine into total RNA in an hour. As shown in Figure 4.3, a 3- to 4-fold decrease in the rate of ^{3}H-uridine incorporation into RNA was observed in the first 12 hr of the *in vitro* incubation. This observation is in agreement with the data presented by Raikow and Fristrom (1971). However, a significant amount of net incorporation was observed in the discs for at least 72 hr.

Due to the fact that there is high content of free amino acids in S medium, this medium is not suitable for studying the ability of imaginal discs to synthesize acid-insoluble protein. Therefore, instantaneous rates of protein synthesis in the discs cultured in S medium were measured by the amount of incorporation of ^{3}H-amino acids into acid-insoluble protein in *Drosophila* phosphate buffer (Robb, 1969) in an hour. The instantaneous rate of protein synthesis, like that of RNA synthesis, decreased rapidly in the first 12 hr of incubation, and then stabilized at an appreciable level for more than 72 hr (Fig. 4.3). The results of this and the previous section indicate that discs can be maintained in S medium for as long as 3 days in a state of biological activity.

Figure 4.2. *Cumulative RNA synthesis in wing discs cultured in S medium.*

Wing discs were cultured in S medium supplemented with 10 μC/ml ^{3}H-uridine. Discs were removed at various times and the amount of acid insoluble radioactivity was determined as described in (B) Experimental Methods.

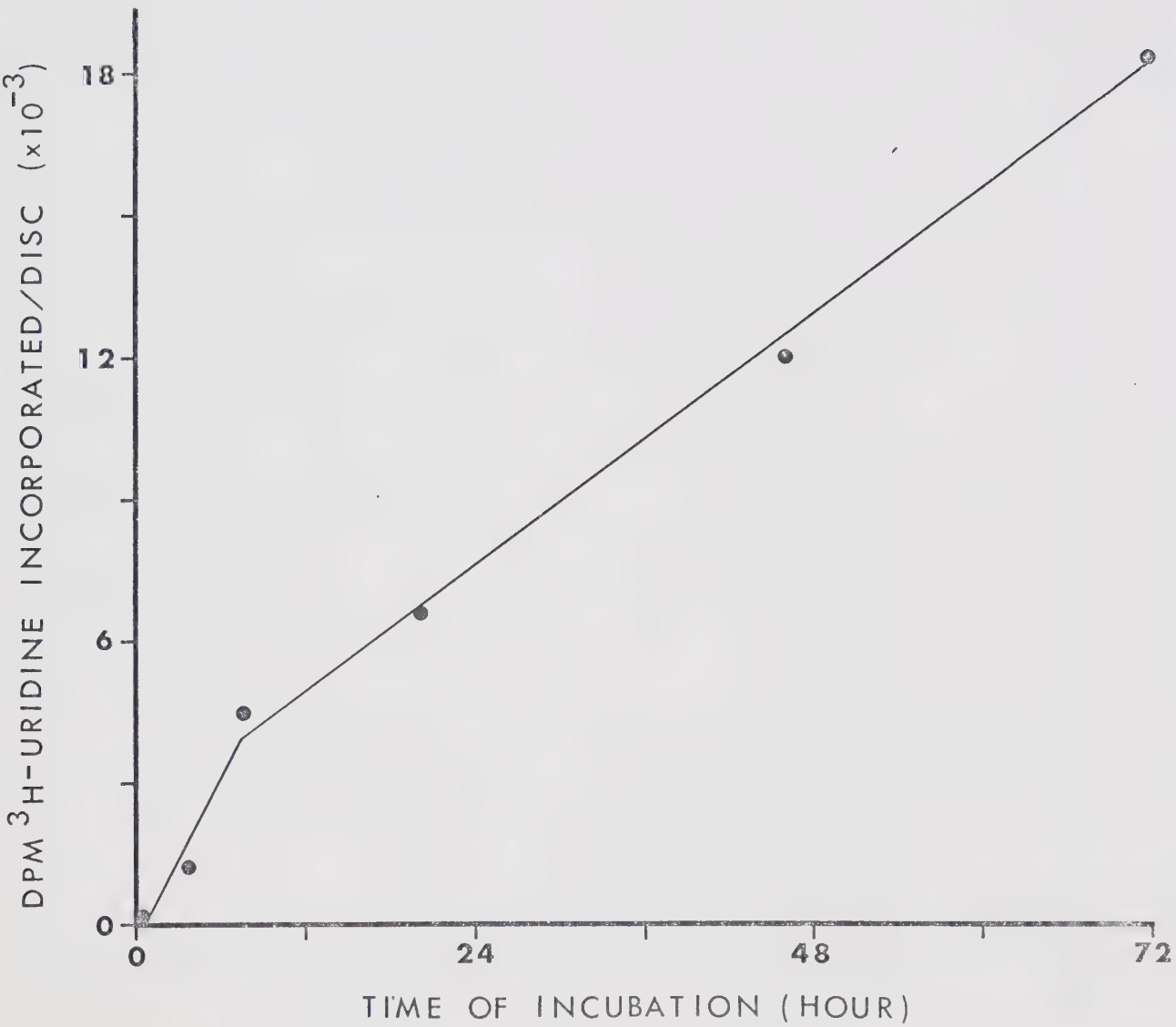
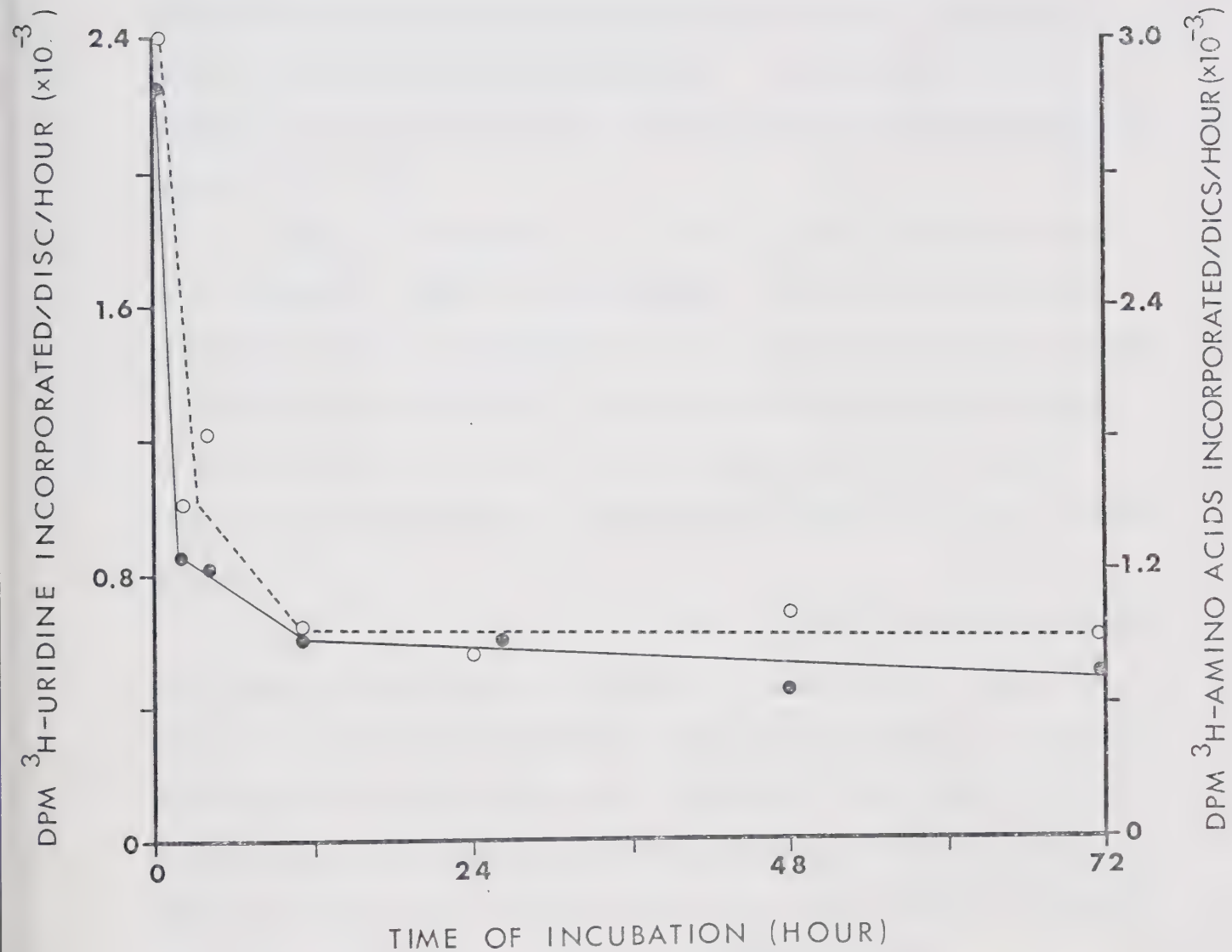

DPM ^{3}H-URIDINE INCORPORATED/DISC ($\times 10^{-3}$)
18
12
6
0
0
24
48
72
TIME OF INCUBATION (HOUR)

Figure 4.3. *Instantaneous rate of incorporation of ^{3}H-uridine, ^{3}H-amino acids into acid-insoluble RNA and protein in wing discs cultured in S medium.*

Wing discs were isolated from synchronized larvae and incubated in S medium at 25°C for various times. Instantaneous net RNA synthesis was measured by transferring the discs to S medium supplemented with ^{3}H-uridine (10 µC/ml) for 1 hr. Instantaneous net protein synthesis was measured by transferring the discs to *Drosophila* phosphate buffer (Robb, 1969) supplemented with a ^{3}H-amino acid mixture (10 µC/ml) for 1 hr.

For each data point, acid insoluble radioactivity was determined in a duplicate pair of discs as described in (B) Experimental Methods.

(—●———●—), RNA synthesis; (···○····○···), protein synthesis.


DPM ^{3}H-URIDINE INCORPORATED/DISC/HOUR (×10^{-3})
DPM ^{3}H-AMINO ACIDS INCORPORATED/DICS/HOUR (×10^{-3})
2.4
1.6
0.8
0
3.0
2.4
1.2
0
0
24
48
72
TIME OF INCUBATION (HOUR)

(iii) *Effect of ecdysterone on incorporation of ^{3}H precursors into RNA and protein in cultured wing discs*

Figure 4.4 presents the effect of ecdysterone on the instantaneous rate of RNA synthesis in wing discs cultured *in vitro*, and the control curve from Figure 4.3 is replotted for comparison. The rate of total RNA synthesis increased sharply from time zero, reached a maximum at about 11 hr and then decreased gradually. Four hours after the rate of synthesis had reached a maximum, the discs showed the first morphological change (Fig. 4.1 b).

The effect of ecdysterone on the rate of protein synthesis in wing discs cultured *in vitro* was also examined. Figure 4.5 indicates the stimulation of the instantaneous rate of acid-insoluble protein synthesis in wing discs by ecdysterone. A comparison of the relative stimulation of protein and RNA synthesis (Fig. 4.6) indicates that the initial stimulation of RNA synthesis is substantially higher than that of protein synthesis.

The sensitivity of the discs cultured *in vitro* to inhibitors of RNA and protein synthesis was also examined. As summarized in Tables 4.4 and 4.5, the ecdysterone-induced RNA and protein synthesis was sensitive to actinomycin D and cycloheximide. Actinomycin D at 1 µg/ml and cycloheximide at 10 µg/ml gave 93% and 100% inhibition of RNA and protein synthesis respectively, and these two concentrations were used as effective inhibitory concentrations in our later experiments.

(iv) *Induction of dopa decarboxylase activity in cultured wing discs by ecdysterone*

Induction of dopa decarboxylase activity was carried out by

Figure 4.4. *Instantaneous rate of RNA synthesis in wing discs cultured in SE medium.*

Wing discs were isolated from synchronized larvae and incubated in SE medium (—●——●—) at 25°C for various times. Instantaneous net RNA synthesis was measured by transferring the discs to SE medium supplemented with ^{3}H-uridine (10 μC/ml) for 1 hr. For each data point, acid insoluble radioactivity was determined in a duplicate pair of discs as described in (B) Experimental Methods.

The control curve (---o----o---) is replotted from Figure 4.3.

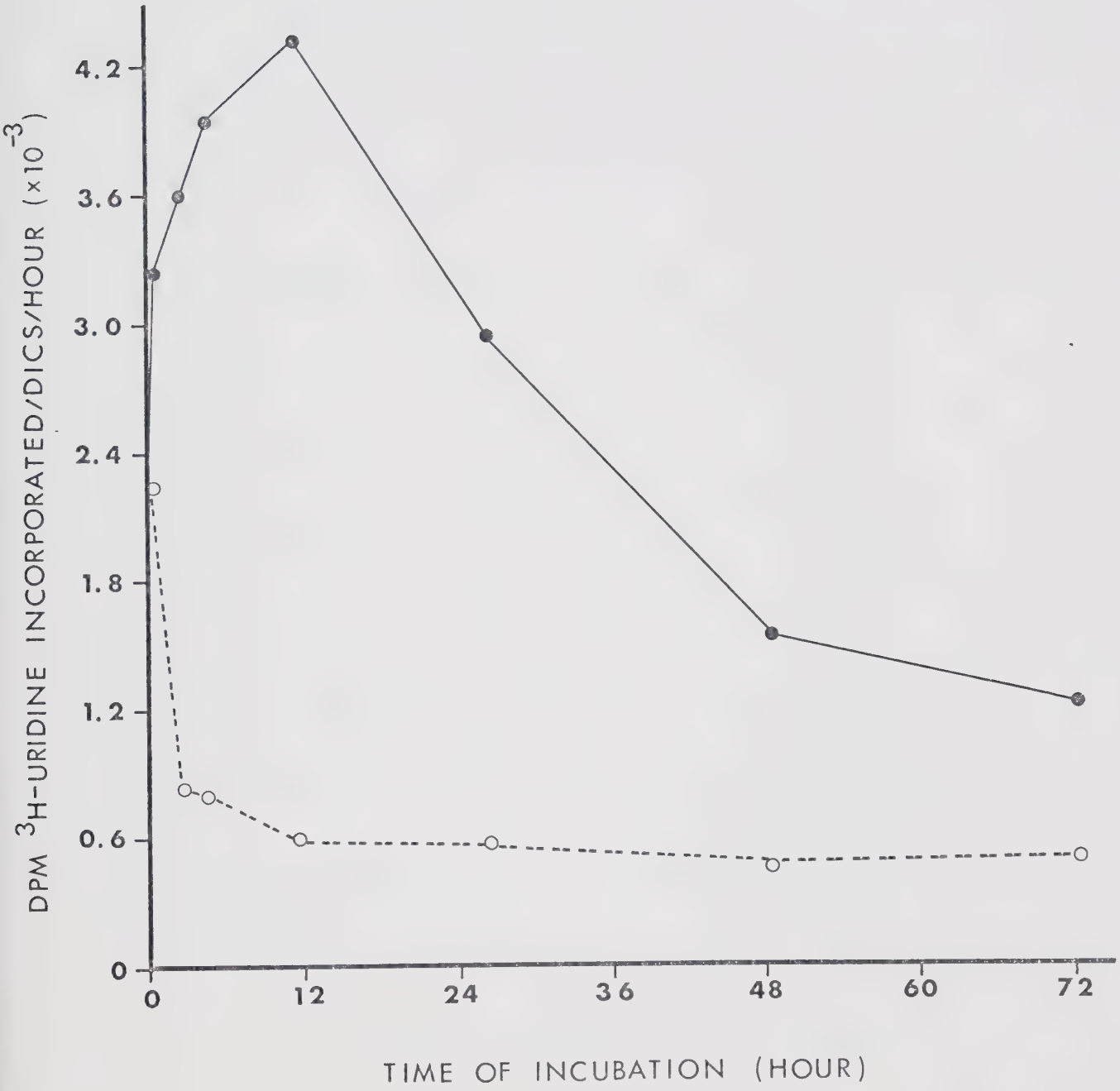

DPM ^{3}H-URIDINE INCORPORATED/DICS/HOUR ($\times 10^{-3}$)
4.2
3.6
3.0
2.4
1.8
1.2
0.6
0
0
12
24
36
48
60
72
TIME OF INCUBATION (HOUR)

Figure 4.5. *Instantaneous rate of protein synthesis in wing discs cultured in SE medium.*

Wing discs were isolated from synchronized larvae and incubated in SE medium (—●——●—) at 25°C for various times. Instantaneous net protein synthesis was measured by transferring the discs to *Drosophila* phosphate buffer (Robb, 1969) supplemented with a ^{3}H-amino acid mixture (10 μC/ml) for 1 hr and ecdysterone (2×10^{-6} M). For each data point, acid insoluble radioactivity was determined in a duplicate pair of discs as described in (B) Experimental Methods.

The control curve (- - -○- - - -○- -) is replotted from Figure 4.3.

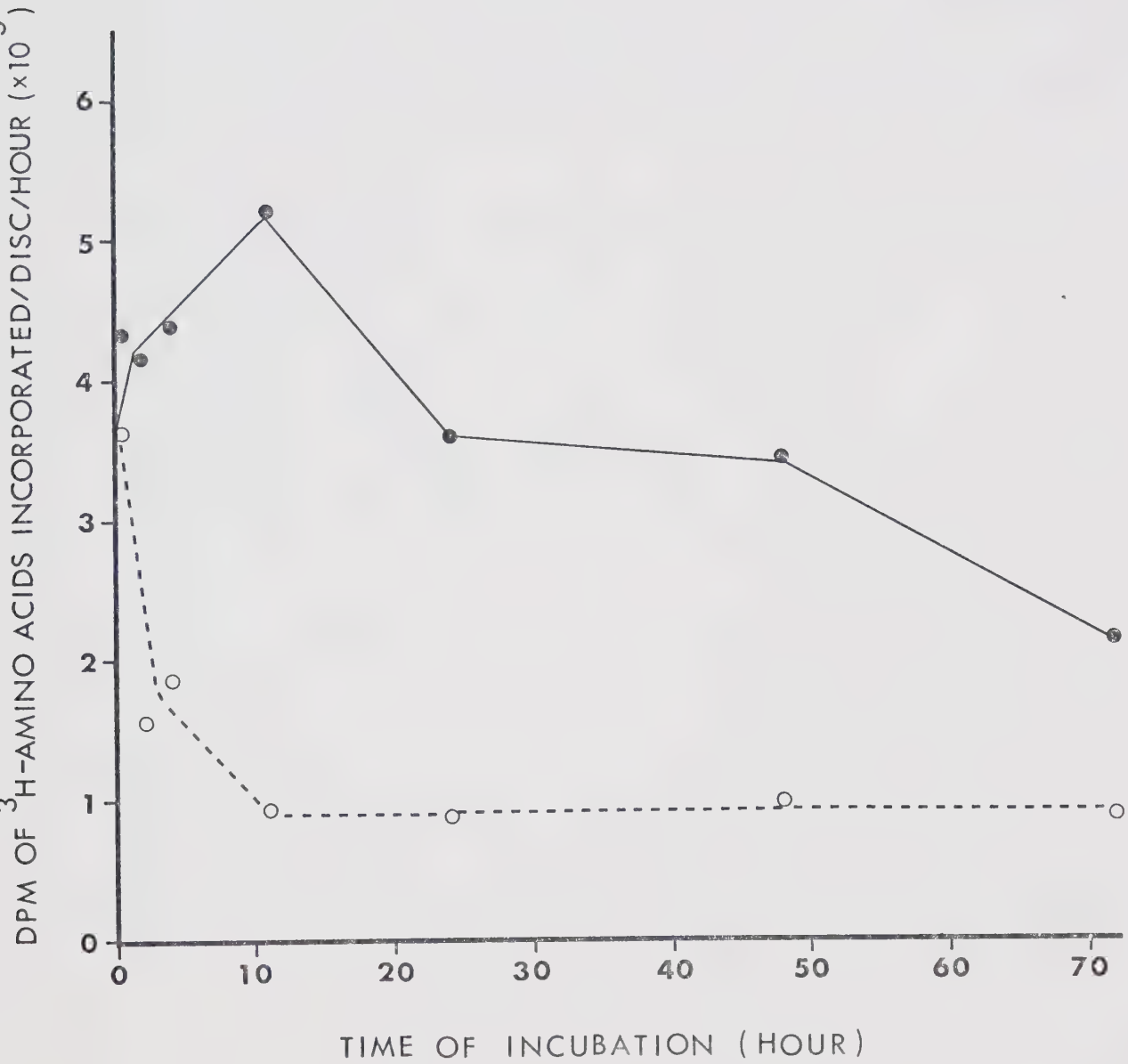

DPM OF ^{3}H-AMINO ACIDS INCORPORATED/DISC/HOUR (×10^{-3})
0
1
2
3
4
5
6
0
10
20
30
40
50
60
70
TIME OF INCUBATION (HOUR)

Figure 4.6. *Comparison of relative rates of instantaneous RNA and protein synthesis in wing discs cultured in SE medium.* (···o····o···), relative rate of RNA synthesis; (—●——●—), relative rate of protein synthesis. The data were obtained by plotting the ratio of the experimental to control curves shown in Figures 4.4 and 4.5.

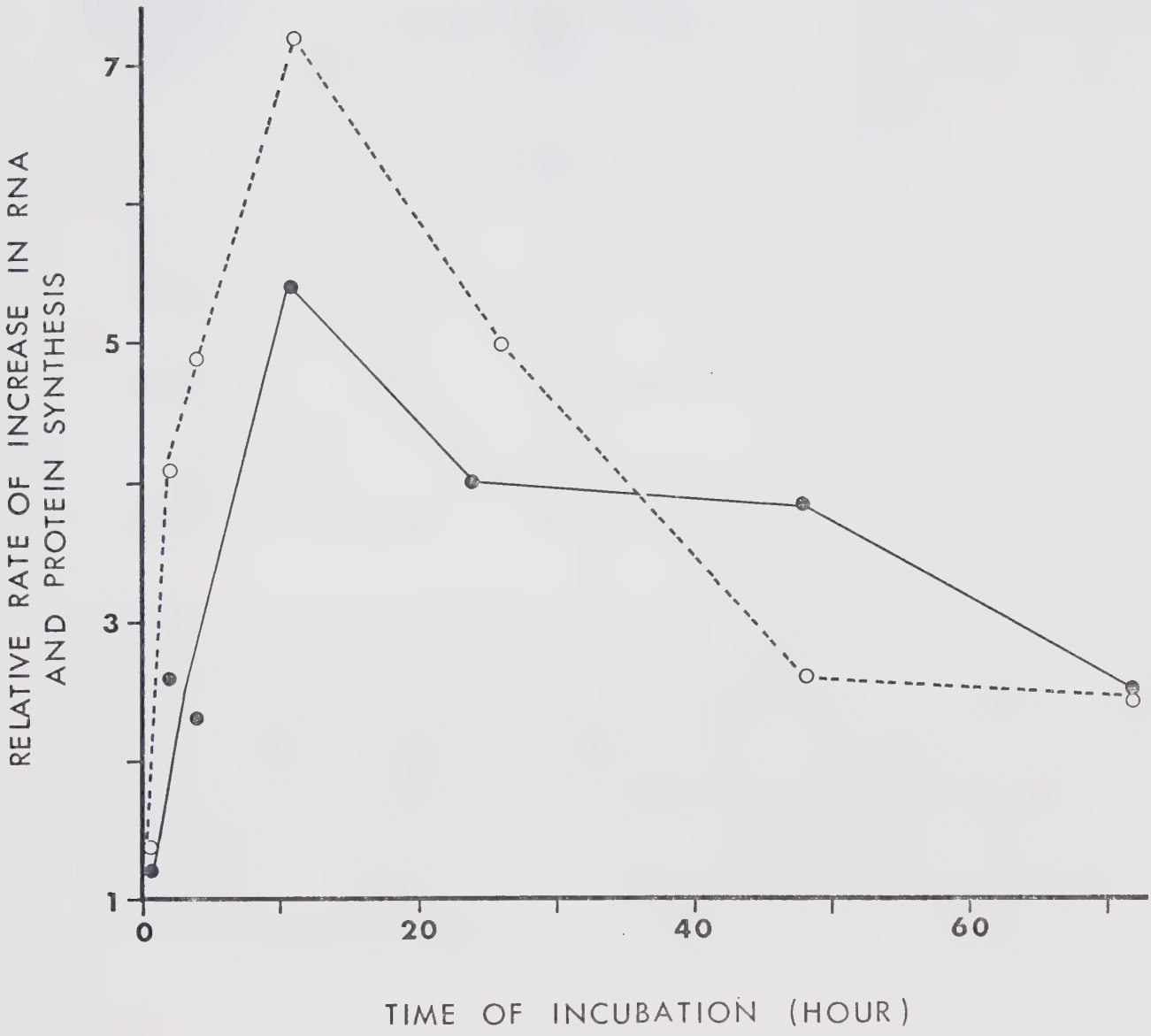

RELATIVE RATE OF INCREASE IN RNA AND PROTEIN SYNTHESIS
7
5
3
1
0
20
40
60
TIME OF INCUBATION (HOUR)

Table 4.4. EFFECT OF ACTINOMYCIN D ON RNA SYNTHESIS IN INDUCED CULTURES OF WING DISCS

Concentration of Actinomycin D (µg/ml)	^{3}H-Uridine Incorporated/Disc/Hour (dpm)	Inhibition (%)
0	1980	0
0.05	330	83.2
0.10	133	93.1
0.50	110	94.4
1.00	150	93.1
5.00	107	94.5

Discs isolated from synchronized larvae were incubated in SE medium with various concentrations of actinomycin D for 4 hr at 25°C. Incorporation of ^{3}H-uridine into total RNA was measured by incubating the discs for 1 hr with 10 µC uridine-^{3}H in SE medium with the same concentration of actinomycin D.

Table 4.5. EFFECT OF CYCLOHEXIMIDE ON PROTEIN SYNTHESIS IN INDUCED CULTURES OF WING DISCS

Concentration of Cycloheximide (µg/ml)	^{3}H-Amino Acids Incorporated/Disc/Hour (dpm)	Inhibition (%)
0	5620	0
0.5	280	95.0
1.0	87	98.5
5.0	0	100.0
10.0	0	100.0
20.0	0	100.0

Discs isolated from synchronized larvae were incubated in SE medium and various concentrations of cycloheximide for 4 hr at 25°C. Incorporation of ^{3}H-amino acids into acid-insoluble protein was carried out in *Drosophila* phosphate buffer (pH 6.8) supplemented with ecdysterone (2×10^{-6} M), the same concentration of cycloheximide and 10 µC of the ^{3}H-amino acid mixture for 1 hr.

incubating wing discs isolated from synchronized larvae in SE medium. The discs were then removed to measure dopa decarboxylase activity at different lengths of time. As summarized in Figure 4.7, after incubation with ecdysterone for 96 hr, enzyme activity was elevated 4-fold relative to the control. Although no hormone was added to the control discs, a substantial level of enzyme activity was observed. This may be due to the presence of a small amount of endogenous hormone in the discs.

The induction of dopa decarboxylase activity requires both RNA and protein synthesis as revealed by studies with inhibitors. As shown in Table 4.6, actinomycin D at a concentration sufficient to inhibit 93% of the RNA synthesis, inhibited 84% of the induced enzyme activity. Cycloheximide sufficient to inhibit all the protein synthesis inhibited 89% of the enzyme activity.

(D) Discussion

The criteria we used to determine the viability of the discs maintained in culture were: (i) the ability of the discs to undergo ecdysterone-induced evagination, and (ii) the ability of the discs to incorporate ^{3}H-uridine and ^{3}H-amino acids into macromolecules. By these criteria, under the culturing conditions we describe, discs isolated from third instar larvae can be maintained in a state of biological and biochemical activity for at least 3 - 4 days.

In their studies on the effect of ecdysone analogues on the *in vitro* development of the wing discs of *Sarcophaga peregrina*, Ohmori and Ohtaki (1973) claimed that ecdysterone at a concentration of 3×10^{-7} M could

Figure 4.7. *Dopa decarboxylase activity in wing discs cultured in SE medium.*

Wing discs isolated from synchronized larvae were incubated in S medium (---o----o---) or SE medium (—●——●—) for various lengths of time at 25°C. For each data point, dopa decarboxylase activity was determined in a duplicate set of 5 discs as described in Chapter II.

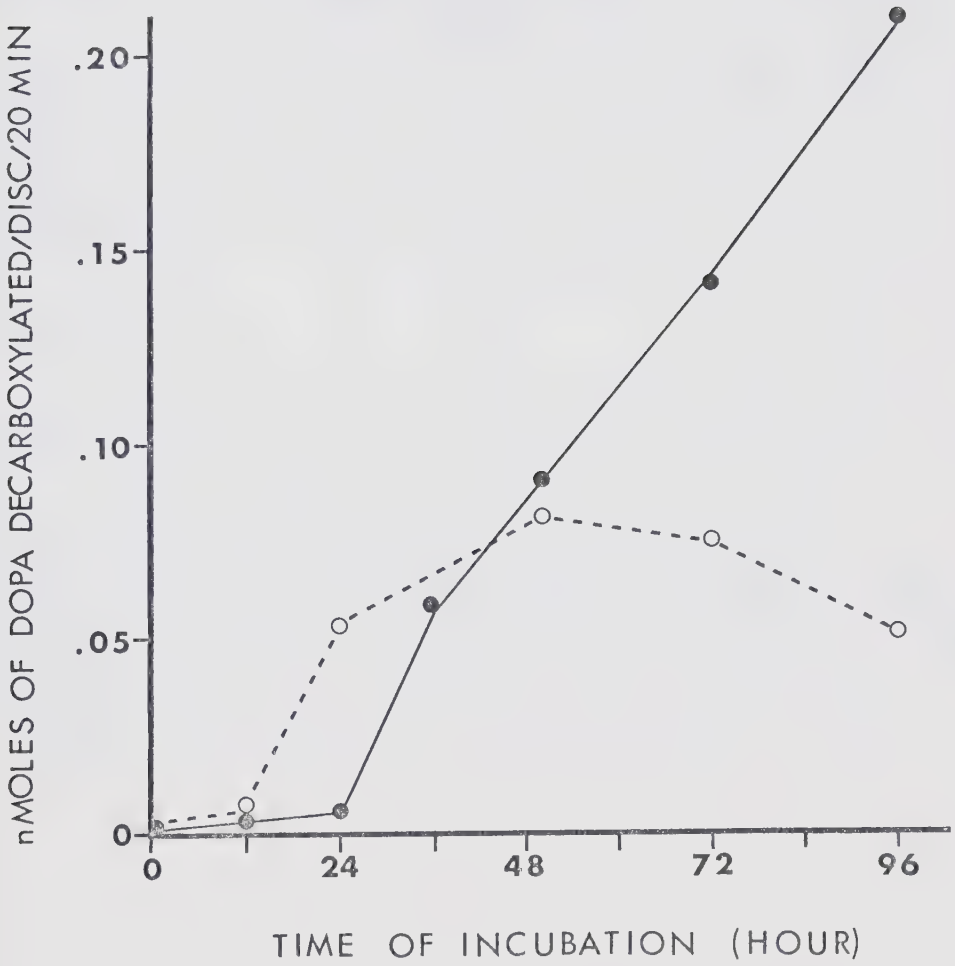

nMOLES OF DOPA DECARBOXYLATED/DISC/20 MIN
.20
.15
.10
.05
0
0
24
48
72
96
TIME OF INCUBATION (HOUR)

Table 4.6. EFFECT OF ACTINOMYCIN D AND CYCLOHEXIMIDE ON THE INDUCTION OF DOPA DECARBOXYLASE IN CULTURED WING DISCS

Conditions	Activity of Dopa Decarboxylase (*n*moles/disc/20 min) ($\times 10^2$)	Inhibition (%)
Control	8.3	0
Actinomycin D	1.3	84
Cycloheximide	0.9	89

The induction was carried out in SE medium for 72 hours. Actinomycin D (1 µg/ml) and cycloheximide (10 µg/ml) were added to the culture at 0 hour. The enzyme activity was measured under the standard conditions in 0.05 M Tris-HCl buffer (pH 7.5).

induce *in vitro* evagination. Inhibition of *in vitro* development was observed when higher concentrations of ecdysterone (over 1×10^{-4} M) were used. Our studies are in agreement with this observation. As Figure 4.1 and Table 4.1 show, ecdysterone ranging from 2×10^{-7} - 1×10^{-5} M induced imaginal wing discs to undergo complete *in vitro* evagination. On the other hand, α-ecdysone at a concentration of 2×10^{-5} M only caused the early morphological changes associated with evagination. Therefore, we conclude that ecdysterone is at least a hundred times more active than α-ecdysone in inducing *in vitro* evagination of imaginal wing discs. Our results are supported by several other investigators. Chihara et al. (1972) reported that ecdysterone is two hundred times more potent than α-ecdysone in inducing *in vitro* evagination of discs isolated from *Drosophila melanogaster*. Similar differences in the activities of these hormones have also been found by Ashburner (1971) for the *in vitro* induction of puffing in the polytene chromosome of salivary glands of *Drosophila melanogaster*. In his study, a concentration of ecdysterone of 10^{-6} M produced the same size puff that was achieved with α-ecdysone at a concentration of 10^{-4} M. Our conclusion is further substantiated by the evidence obtained from the studies on cuticle deposition induced by ecdysterone in cultured cockroach leg regenerates (Marks, 1971), and in cultured wing discs of *Plodia* (Oberlander and Tomblin, 1972). Recently, however, Mandaron (1973) reported that α-ecdysone (6×10^{-7} - 1×10^{-6} M) could induce discs of *Drosophila melanogaster* to undergo complete metamorphosis *in vitro*, whereas, ecdysterone (6×10^{-9} - 2×10^{-5} M) could only induce abnormal and incomplete evagination. The serious discrepancy between

his work and the work reported here and elsewhere, may stem from the fact that his culturing technique involved use of the "hanging drop" method. In any case, further work is necessary to resolve the problem.

Wing discs cultured in S medium incorporate ^{3}H-uridine and ^{3}H-amino acids into total RNA and protein at an appreciable rate at least for 72 hr, although a sharp decrease in the rate of incorporation was observed in the first three hours. As suggested by Raikow and Fristrom (1971), this decrease may be due to the exhaustion of endogenous ecdysterone present in the discs before dissection. This explanation may account for the substantial increase in dopa decarboxylase activity that was observed in the discs, cultured in the absence of the hormone (Fig. 4.7). A longer (2 days) pre-incubation of the discs in S medium would be expected to eliminate both the above effects. This in fact was done by Ohmori and Ohtaki (1973) and as Figures 3 and 6 of their paper show, the sharp decrease in the instantaneous rate of RNA and protein synthesis is eliminated. They ascribe the decrease to the necessity of repairing injuries sustained during dissection.

Finally, we have demonstrated that the activity of dopa decarboxylase can be induced by ecdysterone in discs cultured *in vitro* for 96 hr (Fig. 4.7). This confirms that the discs are a target tissue of ecdysterone. Besides the induction of ornithine decarboxylase in the fat body and wing tissues of silkmoths (Wyatt et al., 1973), dopa decarboxylase is the only other insect enzyme which has been demonstrated to be inducible by ecdysterone. As discussed in the following chapter, the system should prove valuable for further biochemical experiments on the molecular mechanisms of hormone action.

Chapter V

CONCLUSIONS

This thesis describes the biochemical properties of dopa decarboxylase in *Sarcophaga bullata*, the precocious induction of the enzyme in young third instar larvae, and the development of conditions for culturing imaginal discs which made studies on the hormonal induction of dopa decarboxylase possible.

The results presented in Chapter II indicate that the optimum pH for dopa decarboxylase activity in 0.05 M Tris-HCl buffer is 7.5, and the optimum assay temperature is 40°C. Since the reaction mixture was not in contact with the water bath in which the temperature was recorded, the optimum assay temperature we report here does not reflect the actual optimum temperature for the enzyme, which is probably 1 - 5°C lower than that reported. From the studies we reported in Chapter III, it is obvious that pyridoxal-5'-phosphate is an obligatory co-factor for the enzyme. However, the binding between pyridoxal phosphate and the apoenzyme is rather weak, since passage over Sephadex G-200 removed the co-factor. Although the rate of decarboxylation was elevated 40% above the control in the presence of Fe^{+++}, it is not an obligatory co-factor for the partially purified enzyme preparation. The enzyme is rather labile at 4°C, which will present some difficulties in the purification of this enzyme. However, sulfhydryl compounds such as mercaptoethanol and PTU, or pyridoxal phosphate can stabilize dopa decarboxylase activity to a certain extent. The best stabilization conditions are obtained when both mercaptoethanol and pyridoxal phosphate are used,

and therefore it may be advisable to add these two agents to all buffers during purification. Once the enzyme had been passed over Sephadex G-200, it became quite stable (Table 3.5); however, the length of time required for Sephadex G-200 chromatography is a drawback. Therefore, a purification scheme in which an ammonium sulfate fractionation is followed by rapid dialysis on Sephadex G-25, and then chromatography on DEAE cellulose and Sephadex G-200 should be attempted.

During postembryonic development of *Sarcophaga bullata*, three distinct peaks of dopa decarboxylase activity were observed. These occurred at pupariation (peak I, Fig. 3.4), 5-1/2 days after the white puparium stage (peak II, Fig. 3.4) and at adult eclosion (peak III, Fig. 3.4). The enzyme activity at puparium formation was confined to the cuticular epidermal cells which is in agreement with the results of Lunan and Mitchell (1969). The enzyme activity at peaks I and III is associated with the sclerotization of the pupal case and adult cuticle respectively, whereas the enzyme activity at peak II is probably associated with sclerotization of the pupal prothoracic spiracles. The possibility that the activity in peak III is not dependent upon the presence of the molting hormone was raised in the Discussion in Chapter III. Although we regard this as unlikely, until the ecdysterone titer in *Sarcophaga* has been determined throughout development, the question remains unanswered.

The possibility that dopa decarboxylase also performs a neurological function in *Sarcophaga* was not directly investigated. However, the homogeneity of dopa decarboxylase on DEAE cellulose and Sephadex G-200, indicates the enzyme activity probably resides in a single

molecular species. Furthermore, if dopamine is functioning as a synaptic transmitter in *Sarcophaga*, then the dopa decarboxylase which produces this dopamine would be expected in the adult brain. When the enzyme activity in the heads of 10 day old adults was examined on DEAE cellulose, it chromatographed identically to the enzyme present in the cuticular epidermal cells.

In order to provide direct evidence for the contention that dopa decarboxylase activity is regulated by ecdysterone, the *in vivo* induction of dopa decarboxylase was attempted by the injection of ecdysterone in 5 day old larvae. A 4-fold increase in dopa decarboxylase activity over the controls was found 50 hr after the injection of hormone. The enzyme activity induced in such larvae had similar chromatographic properties on DEAE cellulose to the enzyme produced at puparium formation during a normal developmental cycle. Furthermore, at least 60% of the induced enzyme activity was localized in the cuticular epidermal cells. Thus this precocious induction of dopa decarboxylase appears similar to the normal course of events which occurs at pupariation.

However, to study the molecular mechanism of the induction process, it was desirable to develop a system in which epidermal cells could be maintained *in vitro*. The morphological changes that the imaginal discs undergo in the presence of ecdysone, provide a visual key to the induction processes. For this reason, we chose to culture imaginal wing discs rather than the cuticular epidermal cells. As the results presented in Chapter IV show, we have found that wing discs maintained in S medium up to 4 days, retain their ability to synthesize RNA and protein as well as their ability to undergo normal evagination process (shown in Fig. 4.1),

when transferred to SE medium. Since the results clearly showed that ecdysterone has a much higher biological activity than α-ecdysone in inducing evagination, this form of the hormone was used for the biochemical studies.

Both RNA and protein synthesis were stimulated immediately after the addition of ecdysterone, although as shown in Figure 4.6 the initial stimulation of RNA synthesis was substantially greater than that of protein synthesis. Unlike Fristrom (1972), who was studying discs of *Drosophila*, we could not detect any lag in protein synthesis following the administration of ecdysterone. The fact that our discs were hand-dissected in S medium, as opposed to being mass isolated, could well explain the difference in the results. The mass isolation procedure, which requires passing the discs through Ficoll gradients and several rinses with water, could well have reduced the level of protein synthesis in these discs.

Dopa decarboxylase activity in wing discs cultured in the presence of ecdysterone for 96 hr was elevated 4-fold above that in control discs. Actinomycin D and cycloheximide inhibited the induction of enzyme activity, suggesting that hormonally-induced RNA and protein synthesis is a prior requirement for the appearance of dopa decarboxylase activity.

In conclusion, this thesis is the first report of the hormonal induction of dopa decarboxylase in cultured animal tissues. Dopa decarboxylase and ornithine decarboxylase (Wyatt et al., 1973) are presently the only enzymes in insects whose activity has been induced *in vitro* by the molting hormone, ecdysterone. The sequential morphological changes that accompany the *in vitro* development of imaginal discs cultured

in the presence of ecdysterone, provides a series of developmental landmarks to which biochemical correlates may be made. The 48 hr delay in the appearance of enzyme activity after the addition of hormone, is required by the necessity for substantial disc elongation prior to the sclerotization of the pupal cuticle which is eventually secreted by the discs. It is obvious that slight sclerotization of the cuticle secreted *in vitro* does occur once disc elongation is complete, since discs are extremely fragile until 72 hr after the addition of ecdysterone. The dopa decarboxylase activity associated with the sclerotization of the fine pupal cuticle *in vivo* may be included under the shoulder on peak I in Figure 3.4 when pupation occurs. In the absence of any strong evidence to the contrary then, there is no reason to assume that the *in vitro* induction of dopa decarboxylase by the molting hormone is not a normal developmental process which also takes place *in vivo*. Unlike the induction of ornithine decarboxylase in the wing tissues of silkmoths (Wyatt et al., 1973) and the induction of tyrosine animo transferase in rat hepatoma cells (Tomkins et al., 1969), in which the developmental significance of the process is uncertain, the induction of dopa decarboxylase in the developing wing discs may provide a good model system for studies on the hormonal control of a normal developmental process.

This system should allow one to answer several important questions on the induction process currently under investigation. These are:

1) *What is the half-life of the induced enzyme?*
2) *Is the presence of hormone required throughout the induction period? and*
3) *At what stage can RNA and/or protein synthesis be inhibited without preventing the appearance of the enzyme?*

The size of the discs in *Sarcophaga* makes hand-dissection relatively easy and was an important consideration at the time this work was initiated. However, the development of techniques for the mass isolation of discs from *Drosophila* and the report that such discs undergo complete development *in vitro* (Chihara et al., 1972; Fristrom, 1972) now presents the intriguing possibility of extending the present studies to an organism in which sophisticated genetic analysis is possible.

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